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Functional characterization of WRKY75 transcription factor in SA signalling and cell death by using lesion mimic mutants

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Tiivistelmä – Referat – Abstract

Leaf senescence is a developmental and physiological phase in plants to end leaf development. Environment factors such as drought stress, extreme temperature and pathogen threat and internal factors including age and reactive oxygen species induce leaf senescence. Some phytohormones such as jasmonic acid and salicylic acid play a key function in cell death in plants. WRKY transcription factors is known as one of the largest transcription factor family in plants which regulates a variety of plants processes. *WRKY75* which belong to WRKY transcription factors has shown multiple function in plant development like regulation of Pi starvation responses and root development and flowering. In my thesis, I focused on the role of WRKY75 in senescence and stress responses.

WRKY75 was identified as a positive regulator of cell death in Arabidopsis. *WRKY75* can promote salicylic acid biosynthesis by promote transcript levels of *SID2* and also cause hydrogen peroxide accumulation by suppress the transcription of *CAT2.* Hydrogen peroxide and salicylic acid can promote *WRKY75* transcription at the same time. To evaluate the function of WRKY75 transcription factor in SA signalling and cell death, three lesion mimic mutants *acd5, cat2, dnd1* and their corresponding *wrky75* double mutant were used. Interestingly, no different phenotypes were found between *acd5, cat2, dnd1* and their corresponding *wrky75* double mutants in cell death and hydrogen peroxide accumulation detection in Arabidopsis leaves. Meanwhile, marker genes transcription levels were not different in both short day and long day growth condition. However, different phenotypes were observed in botrytis infection.

Based on these results, we formed a hypothesis that gene redundancy could influence genetic characterization of WRKY75. To overcome this problem, *SRDX-WRKY75* chimeric repressor transgenic lines were generated. The SRDX domain act as a dominant negative regulator to suppress WRKY75 target genes. In future research, these new lines can be used to test transcript levels for putative WRKY75 target genes.

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WRKY75, salicylic acid, ROS, SRDX
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1. Introduction

Leaf senescence has been known as a developmental and physiological phase in plants to end the leaf development. Environment factors such as drought stress, extreme temperature and pathogen threat and internal factors including age and reactive oxygen species both can induce leaf senescence. Some phytohormones such as salicylic acid and jasmonic acid also play a key role in cell death in plants. WRKY transcription factors is known as one of the largest transcription factor families in plants that regulates a variety of plants processes. WRKY75 which belong to WRKY transcription factors has shown multiple function in plant development like regulation of Pi starvation responses, root development and flowering. In my thesis, I focused on the role of WRKY75 in senescence and stress responses.

WRKY75 was identified as a positive regulator of cell death in Arabidopsis (Guo et al. 2017). WRKY75 can promote salicylic acid biosynthesis by increased transcription level of *SID2* and also cause hydrogen peroxide accumulation by suppressed transcription of *CAT2*. Hydrogen peroxide and salicylic acid can promote WRKY75 transcription at the same time. To evaluate the further function of WRKY75 transcription factor in SA signalling and cell death, three lesion mimic mutants *acd5, cat2, dnd1* and their corresponding *wrky75* double mutant were generated in previous research. Our aim was to find different phenotypes between lesion mimic mutants and their corresponding *wrky75* double mutants.

2. Literature review

2.1 Leaf senescence and programmed cell death

Leaf senescence is a developmentally complex physiological phase which can be observed in nature in autumn to end leaf development (Buchanan-Wollaston 1997, Lim, Woo et al. 2003). This biochemical and programmed process is the last phase of leaf development leading to leaf cell death and makes nutrition relocate from plant leaves to reproduction seeds to store energy and nutrients (Woo, Chung et al. 2001, Lim, Kim et al. 2007). This common and critical age-depending process can be induced and regulated by multiple factors including environmental

and internal factors. Environmental factors include temperature, humidity, mineral concentration, pathogens and insect's infection and so on. Internal factors include the age of plant, reactive oxygen species (Kaurilind, Xu et al.), plants hormones and so on. (Lim, Kim et al. 2007, Woo, Kim et al. 2013). Programmed cell death (PCD) is a kind of genetically functional controlled process in the development of livings. It is an active and orderly way of cell death regulated by genes. In this way, unnecessary cells or cells are about to be specialized and removed from the body. The cell death in leaf senescence can be known as PCD since it is controlled by many gene programmes (Lim, Kim et al. 2007). Detailed explanations made by Doorn et al. indicated that PCD in plant cells can be identified as autolytic PCD (Vacuolar cell death) and non-autolytic PCD which has been known as necrotic (Van Doorn, Beers et al. 2011). The characters of autolytic PCD can be shown as cell swelling and DNA degradation. In autolytic PCD, little vacuoles were merged into huge vacuoles in the cytoplasm which took place the space of cytoplasm with a number of cytoplasmic organelles disappearance and induced fracture of tonoplast and cell death in Doorn et al. experiment. This process was always accompanied with DNA, protein and some other macromolecules degradation. Non-autolytic PCD, also can be known as necrotic, mainly takes place in response to excessive abiotic stress. The characters of non-autolytic PCD can be shown as shrunken protoplast. Plants can detect the threat of pathogens and insects by plant pattern recognition receptors (PRRs) efficiently and quickly and restrict the spreading of pathogens infection. Furthermore, resistance genes (R genes) in plants which can induce cell death and active defence signaling networks after they detected the effector molecules of pathogen (Hammond-Kosack and Kanyuka 2007, Reape, Molony et al. 2008, Van Doorn 2011, Van Doorn 2011, Zipfel 2014). The process of cell death after recognition of pathogens is referred to as the hypersensitive response. Phytohormones like salicylic acid (SA) and ROS like hydrogen peroxide (H_2O_2) are active regulators of hypersensitive response plants (Coll, Epple et al. 2011).

2.2 The tripartite amplification loop induce leaf senescence

2.2.1 *Salicylic acid and H_2O_2 roles in Arabidopsis*

SA, which formula is $C_7H_6O_3$, is a lipophilic monohydroxy benzoic acid and phenolic acid. It was identified that SA played a critical role of leaf senescence as a key plant stress defence-regulated hormone during the past decades (Alvarez 2000, Blanco, Salinas et al. 2009). Furthermore, plant disease resistance is regulated by SA. Previous studies showed at least two

pathways of SA biosynthesis. The first one is the isochorismate synthase (ICS) pathway which shows a more significant role in Arabidopsis. The second one is phenylalanine ammonia-lyase pathway, which shows a more significant role in rice (Lefevere, Bauters et al. 2020) (Figure.1). In the last few decades, research on plants has revealed a variety of regulated defence mechanisms such as systemic acquired resistance (SAR) and Hypersensitive response (HR). SAR is a type of physiological immunity induced by multiple plant pathogens (Hammond-Kosack and Jones 1996). Cameron's studies had revealed that plants leaves were infected by plant pathogens and then regulated resistance gene signalling which can induce hypersensitive cell death and made cell death in infection site (Gaffney, Friedrich et al. 1993, Cameron, Dixon et al. 1994, Durrant and Dong 2004). SA was shown as an inducer of SAR as an endogenous signal when plant detected the threat of pathogens and insects. An increasing SA accumulation was found after pathogen infection in many plant tissues. Increased endogenous SA content can promote transcript level of many pathogen-associated genes such as *ICS1* and *PRs* and active plant defence mechanisms inducing SAR to protect plants (Morris, -Mackerness et al. 2000).

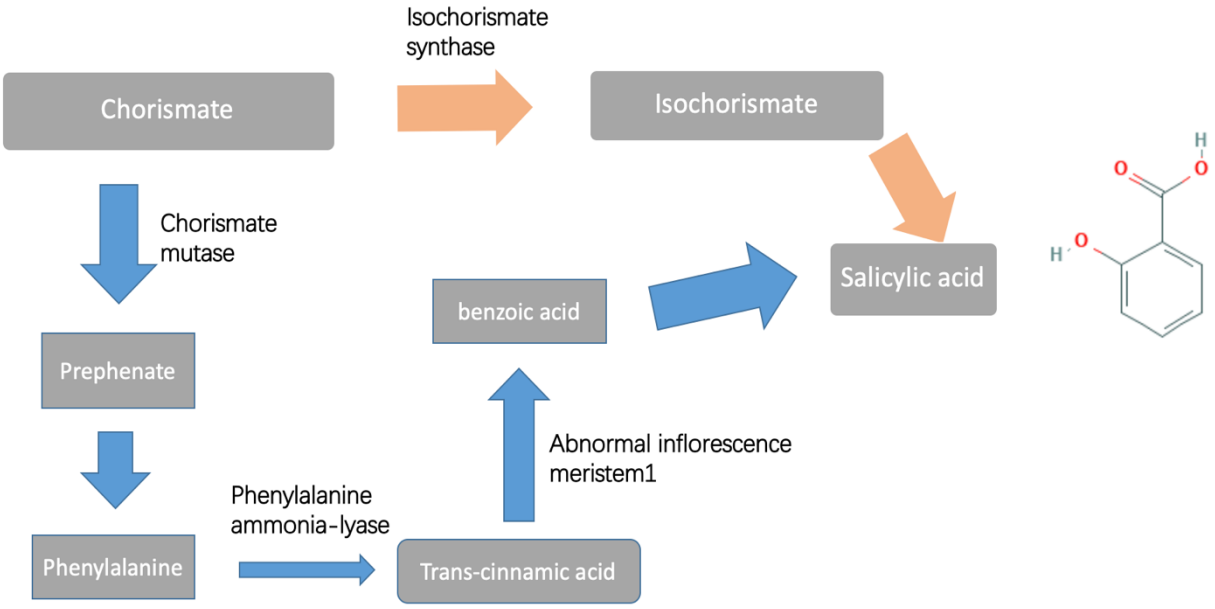

Figure.1: SA molecular structure and two pathway of SA biosynthesis. The orange arrows show ICS pathway and blue arrows show PAL pathway. The enzyme which catalyze Benzoic acid into SA is not identified clearly so far.

Hydrogen peroxide (Figure.2), which formula is H_2O_2, is one of several Reactive Oxygen Species (ROS) which can be manufactured in plants mitochondria, chloroplasts, and

peroxisomes and many other organelles during aerobic metabolism such as photosynthesis (Apel and Hirt 2004, Cho and Seo 2005). In the normal metabolism of plant cells, oxygen molecules act as a significant electron acceptor and some of electrons escaped from the redox system then produce toxic ROS including super anion, singlet oxygen and H_2O_2. H_2O_2 mediated a variety of plant stress response and play a key role in maintaining and regulation of plant stress responses. Increasing levels of endogenous H_2O_2, which induced by plant stress from environment infectors such as extreme temperature, light level, humidity and pathogens and insects infection, can activate pathogen defence regulation, stomatal behaviour and also PCD and leaf senescence (Apel and Hirt 2004, Mittler, Vanderauwera et al. 2004, Cho and Seo 2005). During plant evolution, enzymatic and non-enzymatic systems that maintain the balance of H_2O_2 were formed including Catalase (CAT) (Roschzttardtz, Grillet et al.) regulation system. CAT has a high affinity with H_2O_2 and remove H_2O_2 produced in mitochondrial electron transport and fatty acid oxidation. In photorespiration, plants expended oxygen and release carbon dioxide (CO_2). This process including several enzymatic reactions located in chloroplast, peroxisome and mitochondria (Eisenhut, Roell et al. 2019). In this process, glycolate is generated from 2-phosphoglycolate by phosphoglycolate phosphatase in the chloroplast. Then glycolate was oxidized into glyoxylate with extensive H_2O_2 generation. The H_2O_2 is removed by CAT. So, CAT maintained a certain concentration range of H_2O_2 through its scavenging effect. This protective mechanism of CAT was also reflected in multiple resistance responses of plants. Another H_2O_2 scavenger is ascorbate peroxidase. Overall, H_2O_2 played a key role in inducing leaf senescence, pathogen responses and numerous other stress responses in plants (Durner and Klessig 1995, Rao, Paliyath et al. 1997).

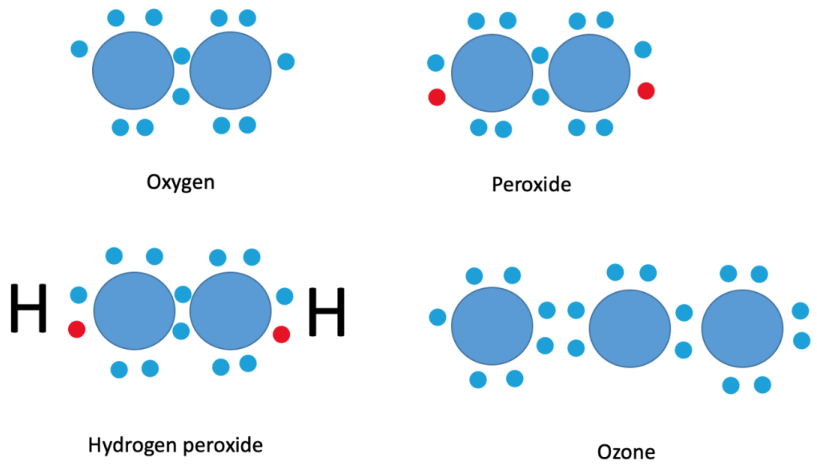

Figure.2: Oxygen and several ROS including hydrogen peroxide structure. Oxygen contains two unpaired electrons, which is equivalent to a double radical. Red circles are electron which oxygen atoms accept.

2.2.2 *WRKY transcription factors family and WRKY75 transcription factor*

Plants are influenced by biotic and abiotic stresses during their developmental period. Biological stress includes attacks from pathogens, fungus and viruses. Abiotic stresses cover soil salinization, drought, heavy metals, extreme temperature, radiation and oxidative stress. Fitting to those pressures and responding to various environmental stresses are pivotal to the plants existing and continuity. Many transcription factor families such as WRKY and ERF are characteristic to plants, and they play a significant and unique role in the regulatory control of plants like leaf senescence (Jiang, Zeng et al. 2012, Woo, Koo et al. 2016). WRKY transcription factors is known as one of the largest transcription factor family in plants which regulates a number of plants processes (Rushton, Somssich et al. 2010). This transcription factor family is defined by a conserved DNA domain called WRKY domain. This DNA binding domain with a length of 60 amino acids is feature-based by a highly conserved WRKYGQK core motif at the N-terminal (Rushton, Torres et al. 1996) (Eulgem, Rushton et al. 2000). Yamasaki 's experiments determined that the WRKY domain in Arabidopsis WRKY4 protein is composed of four-stranded β sheets and has a zinc finger structure formed by Cys/His residues. In addition, a Gly residue in the middle of the N-terminal β chain contributes to the β sheet structure stability (Yamasaki, Kigawa et al. 2005). WRKY transcription factors can be classified into three categories according to the number of WRKY domains and the type of zinc finger structure. WRKY transcription factors which have two WRKY domains are a part of category I. Those belong to category II or III only include one WRKY domain. The zinc finger structure of the members of category I and category II is C_2-H_2 （C-X_{4-5}-CX_{22-23}-H-X_1-H), any amino acid can be X. The zinc finger structure contained in category III WRKY protein is C_2-HC(C-X_7-C-X_{23}-H-X-C) (Li, Xu et al. 2010). WRKY TFs family makes up to 74 members in Arabidopsis playing a main role of plants defence regulation and other internal processes (Pandey and Somssich 2009). Some of them like WRKY6 and WRKY53 are active regulators of leaf senescence compared with WRKY54 and WRKY70 which acts as repressors (Robatzek and Somssich 2001, Miao and Zentgraf 2007, Besseau, Li et al. 2012). Additionally, WRKY75 transcription factor was identified as an activator in leaf senescence (Li, Peng et al. 2012).

2.2.3 *Interaction among WRKY75, ROS and SA in inducing leaf senescence*

Since the function of WRKY75 transcription factor was reported, its regulation has attracted a lot of interest (Figure.3). WRKY75 has been identified as a positive regulator of cell death in plant tissues. *WRKY75* transcript levels can be increased by SA and H_2O_2 and multiple factors (Guo et al., 2017). It has been identified that SA and H_2O_2 induce PCD in plants tissues by mechanisms still to be fully understood. Interestingly, WRKY75 also can promote SA biosynthesis by directly increasing *ICS1* (also known as *SA INDUCTION-DEFICIENT2 (SID2))* transcript levels and suppressing H_2O_2 degradation by repressing transcription level of *CATALASE2 (CAT2)* and suppressed activity of catalases (Guo, Li et al. 2017). Meanwhile, SA and H_2O_2 can accelerate leaf senescence. Taking all results together, an amplification loop model among WRKY75, SA and ROS was presented as inducing leaf senescence in Arabidopsis. However, the exact function of WRKY75 transcription factor in leaf senescence and defence responses require further study.

Function of WRKY75	Reference
A regulator of Pi starvation responses and development in roots	Devaiah et.al 2007
A positive regulator of Flowering in Arabidopsis	Zhang et.al 2018
A positive regulator of cell death in plant	Guo et.al 2017
A negitive regulator of salt and osmotic tolerance	Zhao et.al 2019
An activator of disease resistance	Guo et.al 2017

Figure.3: Several examples of functions of WRKY75 in Arabidopsis. In my case I focused on the role of WRKY75 in senescence and stress responses.

2.3 H_2O_2 and cell death distribution and detection in Arabidopsis leaves.

The location of cell death and ROS accumulation can be visualized with various staining methods in plants. DAB (3,3′-Diaminobenzidine) is known as a derivative of benzene which formula is $(C_6H_3(NH_2)_2)_2$ (Jovicic, Jeftic et al. 2015). This derivative of benzidine is a precursor to polybenzimidazole, which can produce fibers which are famous for their stability in chemistry property (Roschzttardtz, Grillet et al. 2011). When peroxidase is present, DAB is oxidized with H_2O_2 to generate dark brown particles that are insoluble in water (Daudi, Cheng et al. 2012). These brown particles can be used to detect the appearance and accumulation of H_2O_2 in plant tissues. Furthermore, it can be thought as an indirect technique to detect cell death in different plant cells because the H_2O_2 accumulation can active cell death (Kaurilind, Xu et al. 2015).

Trypan blue is a cellular reactive dye and is often used to test the integrity of cell membranes or cell death. When cells are damaged or die, trypan blue penetrates denaturated cell membranes, colouring it. Living cells, on the other hand, keep the dye out. The principle of trypan blue staining experiment is based on the selective transmittance of living plant cell membrane (Wainwright 2010). Selective transmittance means that the membrane can only let some substances (such as glucose, carbon dioxide, etc.) through, and cannot let other substances (such as proteins) through. Selective transmittance is a kind of semi-permeability, and only the bioactive membrane can have selective transmittance. That means the biofilms of living cells are semi-permeable, while the membranes of dead cells are not. Normal alive cells which have intact membrane structure can prevent Trypan blue from entering the cell. In inactive or incomplete cells, the permeability of the cell membrane increases, which can be stained in blue by trypan blue. One of the significant sign of cell death in plants is the loss of cell membrane integrity. Trypan blue staining can be thought as a direct way to detect cell death in plants cells.

2.4 Background of Botrytis

Botrytis cinerea (hereafter Botrytis) is a necrotrophic fungus. It is a typical broad-host-range necrotroph, which kills plant cells to obtain nutrients and causes plants tissues decay. Previous studies have revealed that Botrytis can attack multiple plant species and reduce crop production widely (Elad 1997, Łaźniewska, Macioszek et al. 2010). Necrotic areas with vast fungal growth

are shown and representative appearance of grey mould is given when plants are infected by Botrytis (Govrin and Levine 2000). Botrytis induce ROS accumulation and cell death in Arabidopsis tissues (Birkenbihl, Diezel et al. 2012). Plant defence against Botrytis infection includes pathogen/damage-associated molecular pattern triggered immunity (Mengiste 2012, Lai and Mengiste 2013). Previous studies showed that the pathogen defence pathways in plants are regulated by multiple molecular networks and gene-for-gene interaction (Dangl and McDowell 2006). Multiple resistance responses in Arabidopsis can be induced by Botrytis such as oxidative burst and some genes expression related to plant resistance. Plants resistance to necrotrophic pathogens like Botrytis is always regulated by jasmonate acid (JA) and ethylene (ET) pathway. For instance, some defense gene transcription such as *PDF1.2* transcription can be induced and regulated by JA and ET and then activate botrytis resistance. (Govrin and Levine 2002, Ferrari, Plotnikova et al. 2003). Although SA-regulated-resistance mostly responses to biotrophic pathogens, SA accumulation also modulates JA responses (Spoel, Koornneef et al. 2003). Using single, double and triple revealed that SA control JA defense in the Arabidopsis-Botrytis interaction (Vuorinen, Zamora et al. 2020).

WRKY transcription factors play a significant regulatory function in plants resistance to Botrytis. For instance, WRKY57 targets the promoters of *JAZ1* and *JAZ5* to promote their transcription directly, WRKY70 and WRKY54 regulate the cell wall related defense response negatively, thereby inhibiting the JA signalling pathway and enhancing susceptibility to Botrytis (Jiang and Yu 2016). Contrary to the above three WRKY transcription factors, WRKY3 WRKY4 and WRKY33 positively regulated the resistance to Botrytis in Arabidopsis (Lai, Vinod et al. 2008, Birkenbihl, Diezel et al. 2012). Additionally, ERF and MYB transcription factors can regulate the resistance to Botrytis. For instances, five ERF genes ERF1, RAP1.2, ORA59, ERF5 and ERF56 are all induced by Botrytis and also induced by ET and JA, which enhance Arabidopsis resistance to Botrytis through the ET and JA signalling pathway (Moffat, Ingle et al. 2012, Zhao, Wei et al. 2012, Meng, Xu et al. 2013). Apart from WRKY, ERF and MYB, there are other transcription factors that play a role in the interactions between plants and Botrytis. For instance, GBF1 (G-box BINDING FACTOR1) suppress the expression of CATALASE 2 (CAT2) gene induced by pathogenic bacteria, and positively regulates the expression of PHYTOALEXIN DEFICIENT 4 (PAD4), thus increasing the susceptibility of Arabidopsis to Botrytis (Giri, Singh et al. 2017).

2.5 Gene redundancy and dominant repression of target genes in Arabidopsis.

Gene redundancy is a wide situation in organisms resulting from gene duplication, where duplicated genes regulate or control the same biological function together (Conrad and Antonarakis 2007). In terms of biological development and evolution, gene redundancy can be thought as a benefit for organisms in nature, as it provides a back-up when one homologue genes become unfunctional. For a particular biological function controlled by homologue genes, genes stability is always limited, inevitable failure will occur such as genes become unfunctional. In order to ensure the normal operation of the biological function, a certain number of spare parts (genes) must be prepared, which constitutes the so-called redundancy. Regulation of gene expression without redundancy is fragile and cannot stand the interference of external random events. It must have enough redundancy so as to maintain organism normal biochemical function (Goffeau, Barrell et al. 1996, Kaul, Koo et al. 2000). Nonetheless, from a scientific research point of view, gene redundancy makes it more challenging to identify and characterize homologues genes. For instances, redundant genes may make phenotype selection and analyzing more difficult and unclear when some phenotypes and pathway are regulated and controlled by many homologues genes in plants. To overcome the adverse impact of gene redundancy on characterization of transcription factors, dominant repression of target genes using chimeric repressor can be an efficient method to use (Hiratsu, Matsui et al. 2003).

The amount of genes coding for transcription factors in Arabidopsis genome is almost twice more than genes in animals genomes (Riechmann, Heard et al. 2000). Arabidopsis gene sequences contain multiple and large scales duplicated genes which may cause much gene redundancy (Gauffier, Lebaron et al. 2016). For instances, *RACK1a, RACK1*b and *RACK1c* are three homologues genes of *Receptor for activated C kinase 1* (*RACK1*) in Arabidopsis which regulate plant development together. It has been shown that the function of *RACK1b* and *RACK1c* were equal to *RACK1a* (Guo and Chen 2008). The presence of gene redundancy in Arabidopsis transcription factors influence the identification and functional characterization of transcription factors and their target genes (Meissner, Jin et al. 1999). Dominant repression of target genes by chimeric repressors can be thought as an efficient technique to overcome of gene redundancy within a transcription factor gene family. Chimeric repressor consists of a transcription factor or DNA binding domain and a repression domain that inhibit transcription. In the fusion (chimeric) transcription factor, its specific target genes are repressed (Hiratsu, Matsui et al. 2003). The ERF associated amphiphilic repression (EAR) motif can be used as a

repression domain (Hiratsu, Matsui et al. 2003). When EAR fuses with an active regulator of transcription (transcription factor), the chimeric repressor with EAR plays a strong negative function and repress target genes expression even in the presence of other strong active domains (Ohta, Matsui et al. 2001, Hiratsu, Ohta et al. 2002). To exemplify the utilization of this technology, it was shown that chimeric repressor EIN3, successfully suppressed *EIN3* target genes and showed ethylene insensitive phenotype in Arabidopsis (Chao, Rothenberg et al. 1997). The Arabidopsis *cuc1 cuc2* double mutant showed cup-shape cotyledons in (Aida, Ishida et al. 1997, Szymanski, Jilk et al. 1998). In a first attempt to construct the chimeric repressor with *CUC1* and *CUC2* to suppress target genes, cup-shape cotyledons did not present in transgenic lines. Basic on this situation, they optimized the EAR repressor activity by minimizing the length of EAR sequence and found a new repressor called SRDX which contained 12 amino (LDLDLELRLGFA). Both *35S:CUC1-SRDX* and *35S:CUC2-SRDX* transgenic lines showed formation of cup-shape cotyledons (Hiratsu, Ohta et al. 2002). Thus, the *SRDX* domain can be thought as strong repressor that can be fused to transcription factors and turn them into repressors of transcription.

2.6 Arabidopsis transgenic lines production.

2.6.1 *Agrobacterium-mediated transformation technique in Arabidopsis*

Agrobacterium-mediated transformation (AMT) in Arabidopsis is a common technique to directly produce Arabidopsis transgenic lines which first mention in Bechtold' research which developed a floral vacuum infiltration technique for Arabidopsis to make transformation more stable in 1993 (Bechtold and Bouchez 1995, Wang, Yaghmaiean et al. 2020). Agrobacterium tumefaciens has the ability that they can transfer foreign genes (T-DNA) into a number of host plants and lead to T-DNA recombine into target plants nuclear genome (Zhao, Gu et al. 1999, Tzfira, Li et al. 2004). Because of this character of Agrobacterium tumefaciens, AMT is also used in plant breeding to transfer specific functional genes into plants to generate new characters of plants to increase crops production or resistance for environmental stresses (Hallauer 2011, Prohens 2011). For Arabidopsis, floral dipping is a widely used technique for AMT to generate new transgenic lines because of the simplicity of floral dipping. Previous research revealed that Arabidopsis developing floral tissues can be dipped in solution which consists of agrobacterium, sucrose and Silwet L-77 (Clough and Bent 1998). A number of factors can influence the success

rate of AMT in Arabidopsis such as temperature, humidity, light intensity, the concentration of bacterial inoculum (Clough and Bent 1998).

2.6.2 *Gateway cloning*

Production of transgenic lines is a widespread method to study gene function. Classical cloning methods based on restriction enzymes is time consuming. To overcome these disadvantages of traditional cloning techniques, gateway cloning has been developed, which is a rapid transformation way to transfer a variety of DNA sequences into multiple vectors for gene functional identification and expression of proteins. Gateway cloning technique is based on specific sites recombination derived from phage lambda infection of *E. coli*. In the BP reaction, one target gene fragment with two attB sites recombine into a donor vector (pDONR) with two attP sites (Karimi, Depicker et al. 2007). The recombination between the respective B and P att-sites leads to the excision of the ccdB kill gene (a negative selection marker in pDONR) and the insertion of the target gene and get an entry clone (pENTR) production. pENTR is connected with two attL sites. In a second step, the pENTR can be recombined by LR clonase to a destination vector which is connected with two attR sites. This recombination between the respective attL and attR sites leads to the insertion of target genes into the destination vector. In contrast to traditional cloning technique, gateway cloning can assemble multiple DNA sequences (PCR product or cDNA sequence) in correct order in a specific and stable way during LR recombination and make DNA recombinant constructions simple and rapid (Landy 1989, Karimi, De Meyer et al. 2005).

2.7 Real time quantitative PCR

To analyze gene expression, i.e. transcript levels for selected marker genes, reverse transcriptase real-time quantitative PCR (qPCR) is one choice. qPCR is a method for fast quantification of mRNA transcript levels (Nygard, Jørgensen et al. 2007). In the first step, complementary DNA (cDNA) is obtained from an RNA template by reverse transcription. cDNA is used as a template for qPCR in the presence of a fluorescent DNA probe EvaGreen. EvaGreen bind to the double-stranded DNA from PCR products, then light will be sent out under excitation. The fluorescence intensity increases by the PCR products accumulation (Rajeevan, Vernon et al. 2001). The qPCR machine can detect and measure amplification-associated fluorescence each PCR cycle (Linn et

al.1998). As a result, the machine detects the amount of PCR product by the intensity of the fluorescence.

In this work, five different marker genes were used for qPCR: *CRK37*, *ICS1*, *PR-1*, *JAZ1* and *PLA2A*. There is a large gene family with receptor-like protein kinases (RLKs) in Arabidopsis. In this family, there is a subgroup of cysteine-rich RLKs (CRKs). Transcript levels of CRKs are often stress- regulated, and induced by pathogen infection (Chen, Fan et al. 2004). Overexpression of CRKs can enhance immunity in Arabidopsis (Yeh, Chang et al. 2015). *CRK37* is one of a member of CRK family. Transcript levels for *CRK37* gene increase in leaf senescence in Arabidopsis. SA is a significant mediator during plant defense response, and most SA is synthesized mostly through the *ICS1* and *ICS2* pathway in Arabidopsis (Wildermuth, Dewdney et al. 2001) (Garcion, Lohmann et al. 2008). There is increased expression of genes encoding pathogenesis-related proteins in response to plant pathogen infection (Choudhuri et al. 2014) including *PR-1* (Pieterse, Van Wees et al. 1996). PR proteins such as PR-1 can inhibit fungi infection in plant. JA activates plant defense and senescence in Arabidopsis (Robson, Okamoto et al. 2010). JA can suppress the expression of *CAT2*, leading to H_2O_2 accumulation and then promote leaf cell death via MYC2 pathway (Zhang, Ji et al. 2020). JASMONATE ZIM 1 (JAZ1) is a nuclear-localized protein that control JA signaling. PHOSPHOLIPASE A 2A (PLA2A) is suggested to be a regulator of plant cell death (La Camera et al., 2009). All selected marker genes have increased transcript levels in leaf senescence based on analysis in the Arabidopsis eFP browser (http://bar.utoronto.ca/efp2/Arabidopsis/Arabidopsis_eFPBrowser2.html).

2.8 Lesion mimic mutants

One genetic resource used in Arabidopsis to study PCD is so-called lesion mimic mutants, these are mutants that spontaneously develop cell death lesions (Bruggeman, Raynaud et al. 2015). One way to use these mutants is to make double mutants and determine if cell death or other phenotypes are altered in double compared to single mutants. Three lesion mimic mutants were used in this thesis to evaluate the function of the WRKY75 transcription factor. They were *acd5*, ACCELERATED CELL DEATH 5 (ACD5), *cat2*, CATALASE 2 (CAT2) and *dnd1*, DEFENSE NO DEATH 1 (DND1). ACD5 encodes a ceramide kinase that plays a role in modulating cell death. For *acd5* mutant plants, they can initially grow normally. However, as the plant grows

older *acd5* mutants show cell death and ceramide kinase and SA accumulation in the end (Greenberg, Silverman et al. 2000, Bi, Liu et al. 2014). There would be small restricted lesions (as a PCD phenotype) shown on *acd5* mutant plants leaves when plants were about 5 weeks old (Bi, Liu et al. 2014). CAT2 encodes a peroxisomal catalase. The *cat2* plants accumulate ROS which can active PCD in plant leaves (Kaurilind, Xu et al. 2015). The PCD related phenotypes appear as necrotic lesions in long day condition (Queval, Issakidis-Bourguet et al. 2007). The DND1 encodes a cyclic nucleotide-gated cation channel. The *dnd1* mutants have several phenotypes including spontaneous cell death in a few growth conditions (Xu and Brosché, 2014). Paradoxically, the *dnd1* mutant is also blocked in activation of cell death in response to some pathogens (Yu et al., 1998). In this thesis, the *acd5*, *cat2* and *dnd1* single mutants and corresponding *wrky75* double mutants were used (Figure.4).

Figure.4 : The example of all lesion mimic mutant and their corresponding *wrky75* double mutant. A and B show *acd5* and *acd5 wrky75*. C and D show *cat2* and *cat2 wrky75*. E and F show *dnd1* and *dnd1 wrky75*. G and F show Col-0 and *wrky75* crispr. Lesion mimic single mutant and *wrky75* double mutants have the same visible phenotypes. All plants were grown in short day (SD) growth conditions.

3. Research objective.

My objective was to understand and evaluate the function of transcription factor WRKY75. According to Guo et al., 2017, the WRKY75 transcription factor was suggested to be:

1. A positive regulator of leaf senescence, as knockout or knockdown of the *WRKY75* gene can delay the leaf senescence process.
2. A regulator of SA biosynthesis by activation of *ICS1*/*SID2* transcription.
3. Regulate ROS production via partly represses the transcription of CATALASE2 to promote the accumulation of H_2O_2

In my experiments, I used *acd5, cat2* and *dnd1* single lesion mimic mutants and corresponding double mutants from a cross with *wrky75* crispr mutant to explore the function of WRKY75. Furthermore, the single and double mutants were also subjected to Botrytis infection. I also generated new lines including *SRDX-WRKY75* chimeric repressor lines to suppress WRKY75 target genes to investigate the function of WRKY75. The specific objectives addressed were as following:

1) To find the different ROS and cell death phenotypes between *acd5, cat2* and *dnd1* single lesion mimic mutants and corresponding *wrky75* crispr double mutants by DAB staining and Trypan blue staining.

2) To analyze marker gene transcript levels in *acd5, cat2* and *dnd1* single lesion mimic mutants and corresponding *wrky75* crispr double mutants and also different *WRKY75* overexpression lines growing in different growth condition (short versus long day) to explore function of WRKY75 transcription factor in regulation of defense gene expression.

3) To find different phenotypes in *acd5, cat2* and *dnd1* single lesion mimic mutants and corresponding *wrky75* crispr double mutants and evaluate *WRKY75* function in disease resistance by Botrytis infection.

4) To investigate if there is a gene redundancy for WRKY75, by generating *WRKY75-SRDX* chimeric repressor lines by Gateway cloning and floral dipping and analyze cell death regulated and SA signalling genes transcription level in *WRKY75-SRDX* chimeric repressor lines to elucidate the function of *WRKY75*.

4. Materials and Methods.

4.1 Plant materials and growth condition

All experiments were done with Arabidopsis (*Arabidopsis thaliana*) plants. Arabidopsis thaliana Col-0 (wildtype) and mutants seeds were all from Mikael Brosche. For DAB staining, Trypan blue staining and Botrytis infection, Arabidopsis plant leaves were from eight different plants. They were Col-0, *cat2* (Kaurilind, Xu et al. 2015), *cat2 wrky75*, *dnd1* (Clough, Fengler et al. 2000), *dnd1 wrky75*, *acd5* (Liang, Yao et al. 2003), a*cd5 wrky75*, *wrky75* (crispr 1 allele, Guo et al. 2017). The *acd5, cat2* and *dnd1* double mutants with *wrky75* were made by Mikael Brosche. In addition, three different *WRKY75* overexpression lines were used for qPCR experiments (Guo et al. 2017).

For plants in DAB staining and Trypan Blue staining, after three days germination in darkness in -4 ℃, all plants were grown at 22 ℃/19 ℃, and relative humidity of 50%/60%, under a 12h light/12h dark cycle for one week. Then plants were transferred into 1:1 peat: vermiculite mixture, four seedlings per pot (8 × 8 cm), grown in the same condition for two weeks. Plants grown in the same growth conditions were also used to genotyping to verify the correct genotype of the double mutants.

For plants used in qPCR experiments, plants were grown in short day (SD) condition and long day (LD) condition respectively. For plants grew in short day condition, after three days germination in darkness in -4 ℃, plants were grown at 22 ℃/19 ℃, and relative humidity of

50%/60%, under a 12h light/12h dark cycle for one week, and then transferred plants into 1:1 peat: vermiculite mixture, five seedlings per pot (8 ×8 cm), grown in the controlled environment growth chambers in the same condition for 4 weeks until lesions could be observed clearly (Figure.4). For plants grew in long day condition, after the same time germination and same day/dark cycle with plants grew in SD, and then transferred plants into 1:1 peat: vermiculite mixture, five seedlings per pot (8 ×8 cm), grown in the controlled environment growth chambers under a 16h light/8h dark cycle about 10-13 days until lesions were clear.

For plants in Botrytis infection, after three days germination in darkness in -4 ℃, all plants were grown at 22 ℃/19 ℃, and relative humidity of 50%/60%, under a 12h light/12h dark cycle for one week, and then transferred plants into 1:1 peat: vermiculite mixture, four seedlings per pot (7 ×7 cm), grown in the controlled environment growth chambers in the same condition for 2 weeks and 2 days.

4.2 Genotyping for mutant lines.

4.2.1 Preparation of plant genomic DNA for PCR

Arabidopsis leaves from Col-0, *acd5, cat2, dnd1* and their corresponding *wrky75* crisp double mutant were collected from different plants. Six plants per genotype were used for PCR based genotyping. Leaves were put into labelled screw-cap tubes with glass beads. 630 µL extraction buffer was added into tubes. The tubes were shaken in Precellys with the following program: 3x10 second 6000 rmp with a 30-second break. Then tubes were centrifuged at maximum speed for 5 minutes and 500 µL supernatant was transferred into clean tubes. Next 500 µL isopropanol were added into each tube and mixed by vortexing, then tubes were centrifuged at maximum speed for 10 minutes. The supernatant was discarded. Remaining isopropanol was eliminated by airdrying and 50 µL sterile water was added into tubes to dissolve the DNA.

4.2.2 PCR and restriction enzyme analysis

1 µl of genomic DNA was used as a template for PCR. PCR reactions (30 µl) contained 3 µl 10*PCR (MAGIC) buffer with $MgCl_2$, 0.2 µl 10 mM dNTP, 0.5 µl primer (forward and reverse), 0.1 µl DreamTaq polymerase and 25.2 µl sterile water. Totally 40 PCR cycles were performed.

Each cycle included a 30-second denaturation at 94 ℃, and an annealing (30 seconds) followed by 1 minute of primer extension at 72 ℃.

For restriction digests of PCR products: 34 µl total volume digest reaction contained PCR product 30 µl, restriction buffer 3.4 µl, sterile water 0.5 µl and restriction enzyme 0.1 µl. The mix was vortexed and centrifuged, then were put into 37 degrees temperature room over night for digestion (see below for restriction enzymes used).

The *acd5* (At5g51290) mutant plants were identified by Derived Cleaved Amplified Polymorphic Sequences (dCAPS) markers with the primers (CTGTGCTTCAAGAATATCTTAG, TTGTACAATTGATTAGTTCAGATACG) and restriction enzyme BglII. The *dnd1* (AT5G15410) mutant plants were identified using dCAPS with the primers (TGCAGGCAGTGTTTTGGTT and ATGAGATTAAGAGCAAAACCCGA) and restriction enzyme MboI. The *wrky75* crispr mutant 1 (Guo et al., 2017) was identified with dCAPS using primers CAGTGGACCAAGAAGTGGTCGTGtC and TGCATGGTTTTTCTTTTCAACACAC and restriction enzyme HincII.

After restriction digest, dCAPS markers were separated on poly acrylamide gel electrophoresis (PAGE) gels. For two gels, 1ml 10xTBE, 4 ml MQ water, 4 ml 29 % acrylamide monomer, 5 µl TEMED and 10% APS 120 µl. The TEMED and APS were added into the mix last and the gel poured between 0.75 mm glass plates. The restriction digest products were blent with 3.5 µl loading dye and pipetted into gel and ran under 215V, until the second loading dye band were 1 cm distance from the bottom (about 1 hour and 15 minutes). The gels were put into TBE buffer and stained with ethidium bromide for 10 minutes and photographed.

The *cat2* mutant was a SALK T-DNA allele (SALK_076998, AT4G35090). PCR was done with (ACATTTTGGAGCATTGACTGG and TCTGGTGCTCCTGTATGGAAC) and the tDNA primer Lba TGGTTCACGTAGTGGGCCATCG. PCR reactions were separated on 1% agarose gels.

4.3 DAB staining.

4.3.1 DAB staining solution

First stock solutions were prepared: 200 mM Na_2HPO_4 (mix 20 ml 1 M Na_2HPO_4 with 80ml MQ water); bleaching solution (mix pure ethanol and acetic acid and glycerol as 3:1:1), 1000ml solution was made with 600ml ethanol and 200ml acetic acid and 200ml glycerol. The final DAB staining solution was made by mixing DAB powder and 200mM Na_2HPO_4 solution, Tween 20, and MQ water. The reagent and amount were showed as follows (Table 1.) The DAB staining solution must be prepared fresh before using. Additionally, covering DAB solution bottle with aluminium foil and keep it in darkness was necessary to minimize DAB solution degradation.

Table 1. Component for DAB staining solution reagent added

Component	Volume
DAB powder	300mg
MQ water	270ml
Na_2HPO_4	15ml
Tween 20	150 µL
Total	300ml

4.3.2 Staining leaves

Arabidopsis plants with visible lesions were chosen (the *acd5*, *cat2* and *dnd1* mutants develop lesions as they grow older). The age of plants was around three weeks. Four plants per genotype were stained. After putting plants into tubes, 35-40 ml DAB staining solution was poured into each tube and fully immerse the plants into DAB solution. Covered falcon tubes with aluminum foil immediately. Put tubes into a vacuum chamber, switch on the vacuum and wait until the pressure is 20 and then release the pressure. Repeat the vacuum program for three times, put all falcon tubes into darkness for 4 hours to 8 hours. To destain leaves, pour off DAB staining solution and replace it with the bleaching solution. Next tubes were put into heat incubator, leaving them in 60 degrees for about 20 minutes. The time was flexible depending on the color of solution. The higher temperature could promote bleaching process comparing with lower temperature. To speed up destaining, the bleaching solution was replaced by new bleaching solution.

The DAB stains were documented with a scanner (EPSON Perfection V750 PRO). One plant from each genotype was selected and put in between plastic covers. The overlapping of leaves was avoided to the greatest extent.

4.4 Trypan blue staining.

4.4.1 Trypan blue staining solution

The Trypan blue stock solution was prepared by dissolving 500 g phenol crystals into 500 ml MQ water. Next phenol solution was poured into 2 L flask. Add 500 ml lactic acid (85%) and 500 ml glycerol and 1 g trypan blue powder. Trypan blue stock solution was diluted two times with 95 percent ethanol to make final trypan blue solution.

Destaining solution was prepared with 500 g chloral hydrate powder dissolved into 200 ml MQ water in the fuming cupboard to make the 2.5 g/ ml concentration of Chloral hydrate (CH) solution.

4.4.2 Staining leaves

Plants were put into 50 ml falcon tubes and immersed into trypan blue solution. A water bath was heated in the fuming cupboard and tubes were put into a hot water bath. All leaves were warmed up for 3-10 minutes until ethanol in tubes boils lightly and then tubes removed from the water bath. After staining of leaves was observed, the trypan blue staining solution was removed. The trypan blue solution was disposed into phenol waste. Then all tubes were inverted on paper towels to drain the remaining stain solution and then samples were covered with destaining CH solution and destained for 24 hours to one week until plant tissues were clear. After destaining all plants were stored in 60% glycerol. Plants or leaves were put between plastic sheets and scanned with EPSON Perfection V750 PRO scanner.

4.5 Marker gene transcript level analysis

4.5.1 RNA extraction

Plants for RNA isolation was grown as described above, either in SD or LD (section 4.1). For one biological repeat, five plants per genotype were pooled and frozen in liquid nitrogen. Samples were stored in -80ºC freezer until use.

RNA was isolated by GeneJET Plant RNA Purification Mini Kit (Thermo Fisher Scientific). 500 µL of plant RNA lysis solution supplemented with 10 µL 2-Mercaptoethanol was pipetted into 2 ml Eppendorf tubes. Plants were ground in liquid nitrogen until a tiny powder was obtained. Then plant tissue powder was moved into Eppendorf tube and mixed with a vortex for 20 seconds. Approximately 80-100 mg plant tissue powder was used for RNA isolation. Tubes were incubated for 3 minutes at 56ºC then were centrifuged for 5 minutes in maximum speed. 500 µL supernatant was collected and transferred into clean microcentrifuge tubes. 250 µL 96% ethanol was added after mixing, the solution was added to purification columns. Tubes were centrifuged for 1 minute at 12000 xg. The flow-through was discarded and 700 µL wash buffer 1 was added to the purification column and centrifuged for 1 minute at a speed of 12000x g. The flow-through and collection tube was discarded, and the purification columns reassembled into clean 2ml collection tubes. 500 µL wash buffer 2 was added to purification column then purification column was centrifuged for 1 minute at a speed of 12000 xg. The flow-through was discarded and wash buffer 2 was repeated. All traces of wash buffer were removed from columns through centrifugation of empty columns for 1 min at maximum speed. The purification columns were transferred into a RNase-free 1.5 ml collection tube. RNA was eluted with 50 µL nuclease-free water. The RNA concentration was measured, and RNA was stored in -80ºC freezer.

4.5.2 cDNA synthesis

One challenge in accurate estimation of transcript levels is that most RNA isolation methods lead to some level of genomic DNA contamination. The first step for cDNA synthesis was DNasel treatment to remove genomic DNA contamination. DNAsel digest single- and double-stranded DNA. The second step was to inactivate DNasel by EDTA and heat treatment. The third step was synthesizing cDNA. 2000 ng RNA was used for DNAseI treatment and cDNA synthesis. If RNA concentration was very low, slightly less RNA was used – but always the same amount for all genotypes in a biological repeat. The RNA was DNAseI treated in a final

volume of 20 µL. For the composition of DNAseI reaction **(see supplementary data 1)**. Tubes were put into PCR machine for 30 minutes in 37 degrees to degrade DNA. Then 2µL 50 mM EDTA was added into each tube and kept them at 65 degrees for 10 minutes to inactive DNaseI. A mastermix **(see supplementary data 2)** that include buffer, dNTPs, reverse transcriptase (Maxima, ThermoFisher Scientific) and RNAse inhibitor (ThermoFisher Scientific). 9.5µL mastermix was added to each tube and incubated in 50 degrees for 2 hours for cDNA synthesis. cDNA products were diluted with adding 70 µl water to a final volume of 100 µl.

4.5.3 Gene expression analysis by qPCR

qPCR was performed with five marker genes and three reference genes (**supplementary data 3** for primer sequences, AGI codes and primer amplification efficiencies). The qPCR was performed in three biological repeats with 5x HOT FIREPol EvaGreen qPCR Mix Plus (Solis Biodyne). Marker genes transcription levels in both SD plants and LD day plants were analyzed in three biological repeats.

Three technical repeats were used for each primer-cDNA combination. Each technical repeat was 10 µL, thus for three technical repeats, a mastermix with 31 µL was prepared. 31 µL total volume contained 6µL 5xEvaGreen, 3µL template, 1.5µL primer and 20,5µL sterilized water. Samples were pipetted into qPCR 384 well carefully. Tips were on the middle of inside surface of wells tightly and samples were pipetted gently and carefully in case samples go out from wells. See **appendix 1** for the layout of the 384 wells plate for qPCR. The qPCR plate was centrifuged at a speed of 4000rpm for 10 min. qPCR plate was put into Bio-Rad CFX384 with cycle conditions in **Table 2**. Melting curve analysis was performed to identify that single PCR products were amplified; and the data were exported and analyzed with Qbase+ software. Primer amplification efficiencies were provided by Mikael Brosche.

Table 2 .qPCR reaction condition

Temperature		Time
95 degree		10:00
95 degree		0:10
60 degree	59 cycles	0:10
72 degree		0:30

measurement of fluorescence	
95 degree	0:10
65 degree	0:05
95 degree	0:50

4.6 Botrytis cinerea infection

For Botrytis infection, the following genotypes were used Col-0 (Wildtype), *wrky75* crispr, *dnd1, acd5, cat2* and their double mutant with *wrky75, dnd1 wrky75, acd5 wrky75, cat2 wrky75*. Botrytis sensitive line *coil-16* was use as a control. Botrytis was provided by Katariina Vuorinen. Arabidopsis were grown for 3 weeks and 2 days weeks under a 12-h light/12-h dark cycle as mentioned above. Then all plants were transferred into mini greenhouses. Botrytis Infection solution was made by dissolving 12 grams PDB power into 1 liter MQ water to make the solution in 1/2 strength and sterilized. Grey Botrytis (spores and hyphae) was selected from the medium with sterile forceps. The grey Botrytis was put into a 2 ml Eppendorf tube filled with infection solution. The Tube was vortexed for 3-5min to break the mass. Then the mix was filtered through a two-layer microcloth which should be wet before filter the mix into a new tube. The filtered solution was prepared to be diluted for spore calculations if the solution looks dark. All filter steps were in sterile conditions. A microscope and a counting chamber were used to count the spore number. The aim was to find about 12 spores in one small square to get a reliable spore count. The spore count was to be calculated until 1 million spores per milliliter of half-strength PDB solution.

Then 5 µl botrytis drops were used to infect plants. 4 leaves of per plants were infected by putting Botrytis solution on the upper leaves side. Mini greenhouses were sealed with tapes after Botrytis infection, and enough moisture was added before sealing (spraying the inside of the minigreenhouse roof by water). In order to active diseases, plants were kept under a higher humidity in a mini greenhouse with (21 degrees) day and (18 degrees) night temperature in a 12h light/12h dark cycle. Photos of infection sites were taken 48 – 72 h after infection and lesion sizes measured**.** Anova test and T-test were used to investigate the significance difference between Col-0, *acd5, dnd1, cat2* and their corresponding wrky75 mutant. Three biological repeats were done for Botrytis infection.

4.7 Construction of Plasmids and Transgenic Plants Generation for WRKY75-SRDX line

4.7.1 Cloning of WRKY75 - SRDX.

The aim was to make four variants of WTKY75- SRDX chimeric repressor in vectors suitable for Agrobacterium-mediated transformation of Arabidopsis plants. They were respectively *WRKY75* cDNA: wildtype SRDX with 35S promoter, *WRKY75* cDNA: mutant SRDX with 35S promoter, *WRKY75* genomic DNA: wildtype SRDX with native promoter and *WRKY75* genomic DNA: mutant SRDX with native promoter. The mutant versions were made to inactivate the SRDX domain to be used as controls for the wildtype versions. PCR and Gateway cloning was used for plasmid construction.

To construct *WRKY75* genomic DNA: wildtype *SRDX*, $attB_1$:native promoter:*WRKY75* genomic DNA:*SRDX* and *SRDX*:*WRKY75* 3'UTR:$attB_2$ were constructed first. The $attB_1$:native promoter:*WRKY75* genomic DNA: *SRDX* product was produced by PCR (Forward primer is attB1: *WRKY75* promoter, reverse primer is *SRDX*:*WRKY75*). The template was Col-0 genomic DNA. The *SRDX*:*WRKY75* 3'UTR:$attB_2$ was produced by PCR (Forward primer is *SRDX*: *WRKY75* 3'UTR, reverse primer is *WRKY75* 3'UTR: $attB_2$). The template was *WRKY75* cDNA. Then two products were reassembled by PCR and $attB_1$:native promoter:*WRKY75* DNA: wildtype SRDX:*WRKY75* 3'UTR:$attB_2$ was produced. The $attB_1$: native promoter:*WRKY75* cDNA: wildtype SRDX:*WRKY75* 3'UTR:$attB_2$ and $attB_1$:native promoter:*WRKY75* DNA: mutant *SRDX*:*WRKY75* 3'UTR:$attB_2$ and $attB_1$: promoter:*WRKY75* cDNA: mutant *SRDX*: *WRKY75* 3'UTR:$attB_2$ were produced in the same way (Figure 5).

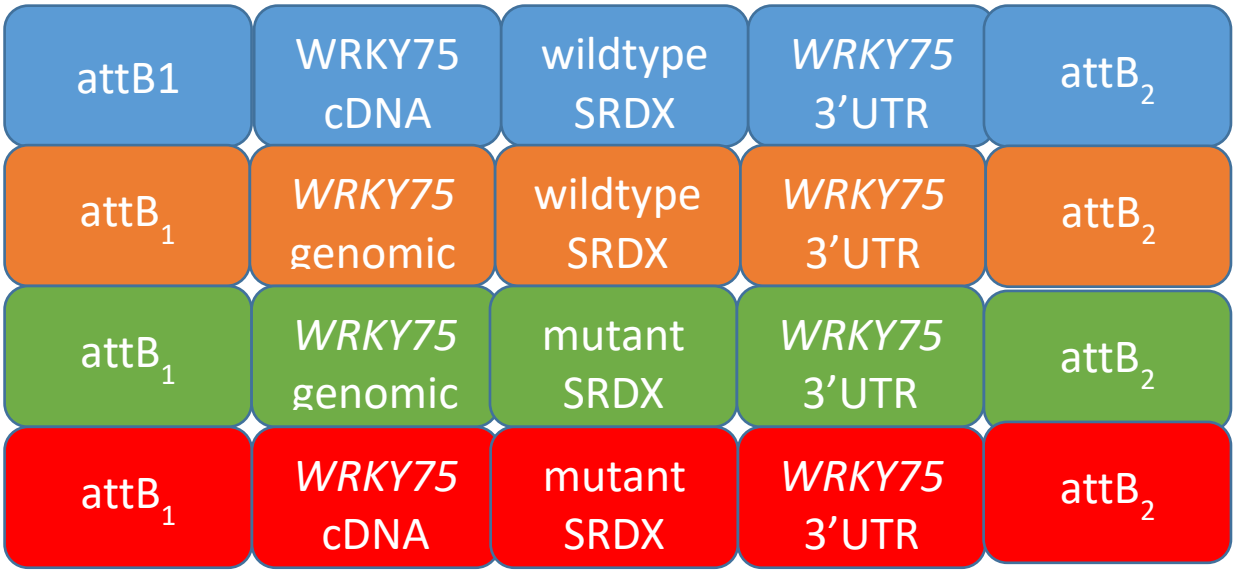

Figure.5 :_Four different *WRKY75-SRDX* contructs were produced with PCR which were transferred into an pDONR/Zeo entry vector.

The final PCR product was recombined to pDONR/Zeo Entry vector with BP clonase in the room temperature overnight. 1 µl product was used to transform 50 µl competent DH5α *Escherichia coli* cells by heat shock. Liquid LB medium was made then cells were incubated on a shaker in 37 degrees for 1 hour. Then 100 µl product was plated on the LB medium and incubated in 37 degrees overnight. Positive clones were identified and sequenced.

Subsequently, the WRKY75-SRDX genes were transferred to destination vector with LR clonase by LR reaction. Two destination vectors used were pMDC32 (with 35S promoter) and pMDC100 (for the native WRKY75 promoter). pMDC32 confers hygromycin resistance and pMDC100 confers kanamycin resistance in plants (Curtis and Grossniklaus 2003). All DNA products were verified by PCR and electroporated into Agrobacterium. Agrobacterium was used to transform Col-0 and *wrky75 crispr* mutant by floral dip.

4.7.2 Generation of WRKY75-SRDX Transgenic Plants by floral dip method

Single Agrobacterium colonies were grown overnight in 5 ml LB + 100 µg/ml of kanamycin, 50 µg/ml of gentamycin and 20 µg/ml of rifampicin. Next day 1 ml culture was inoculated to 100 ml LB + 100 µg/ml of kanamycin, 50 µg/ml of gentamycin and 20 µg/ml of rifampicin. Agrobacterium was harvested by centrifugation 15 minutes at 4000 rpm. The Agrobacterium pellet was resuspended in 100 ml 5% sucrose and 0.05% Silwet L-77. Flowering Arabidopsis plants were dipped into Agrobacterium and left at 100% humidity overnight. The next day plants were transferred to new trays and grown until seeds can be harvest.

The following constructs were used to transform Arabidopsis (pMDC 32:*WRKY75* cDNA: wildtype *SRDX*; pMDC 32: *WRKY75* cDNA: mutant *SRDX*; pMDC100: *WRKY75* genomic DNA: wildtype *SRDX*; pMDC100: *WRKY75* genomic DNA: mutant *SRDX*). The constructs were transferred into both Col-0 and *wrky75 crispr* mutant.

4.7.3 Transgenic line selection with hygromycin and kanamycin

Selection was done on ½ MS medium. To get enough medium, 5 L was prepared: 11g MS salt was dissolved 5 L water and pH adjusted to 5.7 with KOH (5 M or 0.5 M as needed). 3.5 grams agar were added into ten 500 ml bottles, respectively. 500 ml MS-medium was added to each bottle and sterilized. Hygromycin (final concentration 20 μg/ml) or kanamycin (50 μg/ml) was added for selection of transformed seeds.

Seeds were sterilized with ethanol. Seeds were put into 2 ml tubes. 500 μL 70% ethanol with 2% Triton X-100 was added into tubes and shaken gently for 10 minutes on a shaker. Then seeds were washed by 99% ethanol two times. Seeds were pipetted and dried on filter paper. The seeds with pMDC32 were put on the hygromycin selection plates, and seeds with pMDC100 were put on the kanamycin selection plates. Col-0 was also put into both mediums to act as a control for selection. To synchronize germination plates were kept at 4 degree dark place for two days. Kanamycin plates were put into a chamber with (21 ℃) day and (19 ℃) night temperature with a 12-h light/12-h dark cycle for 10 days. Hygromycin plates were put into the same chamber in light condition for 8 hours and then were transferred into darkness (22 ℃) for 48 hours. Then all Hygromycin mediums were transferred into the same chamber for 10 days. Resistant seedlings were transformed into soil and grown for seed production.

T1 plants were harvested and seeds were collected after plants were mature. The seeds (T2) were selected again on MS-medium with kanamycin and hygromycin selection as described above. According to Mendel's laws, the ratio of kanamycin resistance seedlings number to sensitive seedlings would be 3:1 if there is one T-DNA insertion. The number of kanamycin resistance and hygromycin resistance seedlings were counted and seedlings were transferred into soil from lines with a segregation ratio about 3:1.

Selection of suitable lines was further tested by evaluation of expression of the WRKY75-SRDX transgene with qPCR. As the native WRKY75 promoter has very low expression in control conditions, these lines were treated with ozone to strongly activate the promoter. Ozone treated plants (350 ppb, 2h) and control plants were collected for RNA extraction, cDNA synthesis and qPCR. The following primers were used for qPCR to detect the expression of WRKY75-SRDX (Forward primer: GTGGACCAAGAAGTGGTCGT and reverse primer CAGTTCCAGATCCAGATCCA).

5. Results

5.1 Genotyping results of all mimic mutant lines used.

The aim of the genotyping was to identify that the mutants used in this work were correct. Genotyping was performed first with *acd5*, *dnd1* and *cat2* markers. Subsequently, the *wrky75* crispr mutation was checked with the *wrky75* dCAPS marker (restriction enzyme HincII).

Homozygous *acd5* mutant plants were identified with *acd5* dCAPS and digested with BglII. Both *acd5* and *acd5 wrky75* were cut by restriction enzyme BglII which cut *acd5* mutant (Figure 6). Subsequently, with the *wrky75* dCAPS marker the *acd5* single mutant and Col-0 was digested by Hincll, while *acd5 wrky 75* was not digested by Hincll showing that the double mutant was homozygous for *wrky75*.

In Figure 6 C and D, with the *dnd1* dCAPS marker, *dnd1* and *dnd1 wrky75* were digested by MboI while Col-0 was not digested. For the *wrky75* dCAPS marker, the *dnd1* single mutant and Col-0 were digested by Hincll, while *dnd1 wrky75* was not digested (Figure 6). This shows that the *wrky75 dnd1* double mutant is homozygous.

The *cat2* tDNA mutant was genotyped with a three primer PCR reaction (two gene-specific primers LP and RP, + a tDNA primer). If a plant is homozygous for *cat2*, the expected result is a short band in *cat2* and a longer PCR product in wildtype (heterozygous plants will have both products). Both *cat2* and *cat2 wrky75* were homozygous for *cat2* (taking into account that two *cat2 wrky75* samples failed). With the *wrky75* dCAPS marker *cat2* and Col was digested, but the *cat2 wrky75* was not digested by HincII, confirming that *cat2 wrky75* is homozygous.

Finally, the single wrky75 crispr mutant was also confirmed to be homozygous. These results indicated *acd5* and *acd5 wrky75* mutant, *dnd1* and *dnd1 wrky75* mutant, *cat2* and *cat2 wrky75* and *wrky75* crispr mutant were all homozygous correct lines.

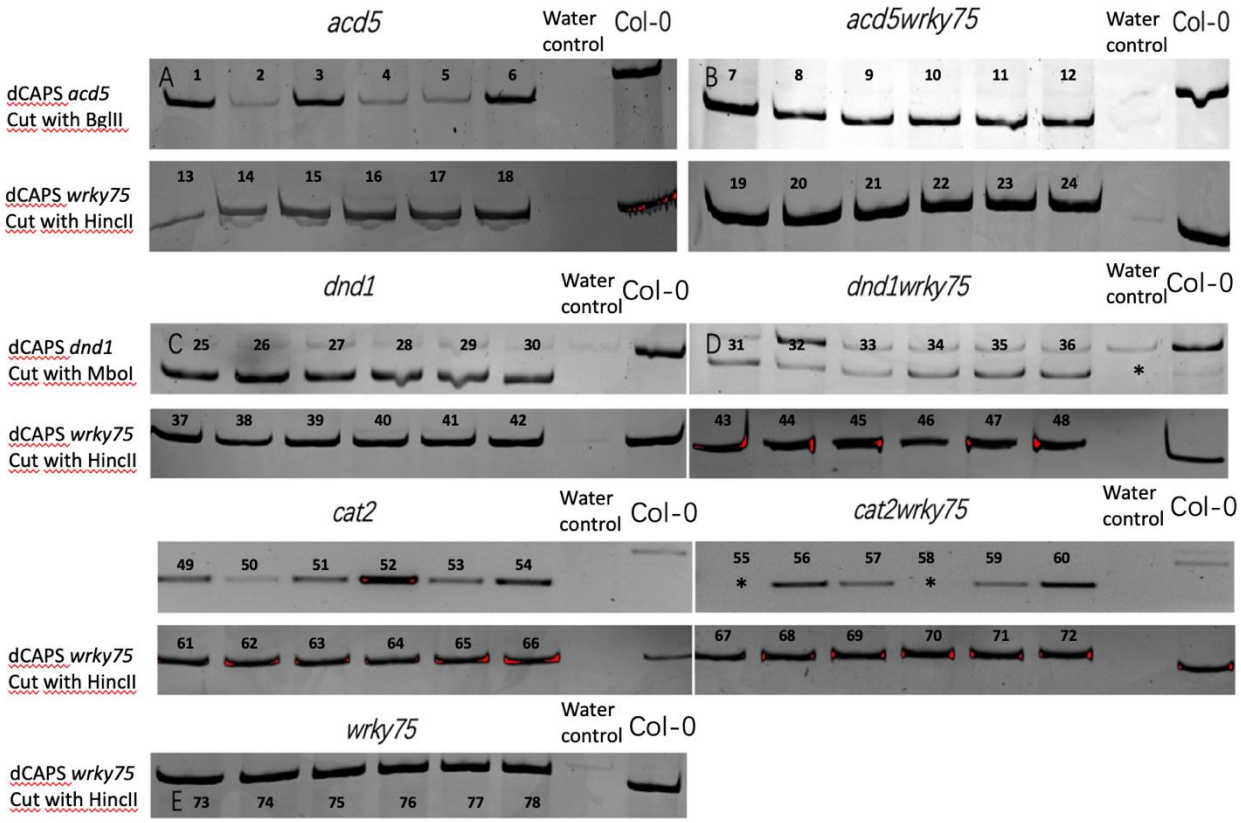

Figure.6: Single and double mutants were genotyped with PCR based markers. For *acd5*, *dnd1* and *wrky75* dCAPS markers were used and after PCR, the products were digested with BglII, MboI or HincII, respectively. The *cat2* mutants is a tDNA allele and was genotypes with a three primer reaction with LP and RP gene specific primers and Lba tDNA primer. * indicate failed PCR reactions. Note that the MboI enzyme was old and did not fully cut the *dnd1* PCR product. Both *dnd1* and *cat2* have distinct growth phenotypes (Figure 4), which were the same in the corresponding *dnd1 wrky75* and *cat2 wrky75* double mutants. This distinct growth phenotype also shows that the mutants are homozygous.

5.2 DAB staining

With DAB staining, the accumulation of H_2O_2 was monitored in the various genotypes. DAB stain displayed that there were some brown particles in *acd5* and *acd5 wrky75* (Figure 7), which meant that in both *acd5* and *acd5 wrky75* double mutant phenotype there was H_2O_2 accumulation. Additionally, *cat2* (Fig.7) mutant displayed some brown areas which mean H_2O_2 accumulated in *cat2*. No dark brown precipitate was seen in *dnd1* single mutant and *dnd1 wrky75* double

mutant. Additionally, no dark brown precipitate was seen in wildtype and *wrky75* single mutant. This experiment showed that H_2O_2 accumulated in *cat2*, *cat2 wrky75*, *acd5*, *acd5 wrky75* plants.

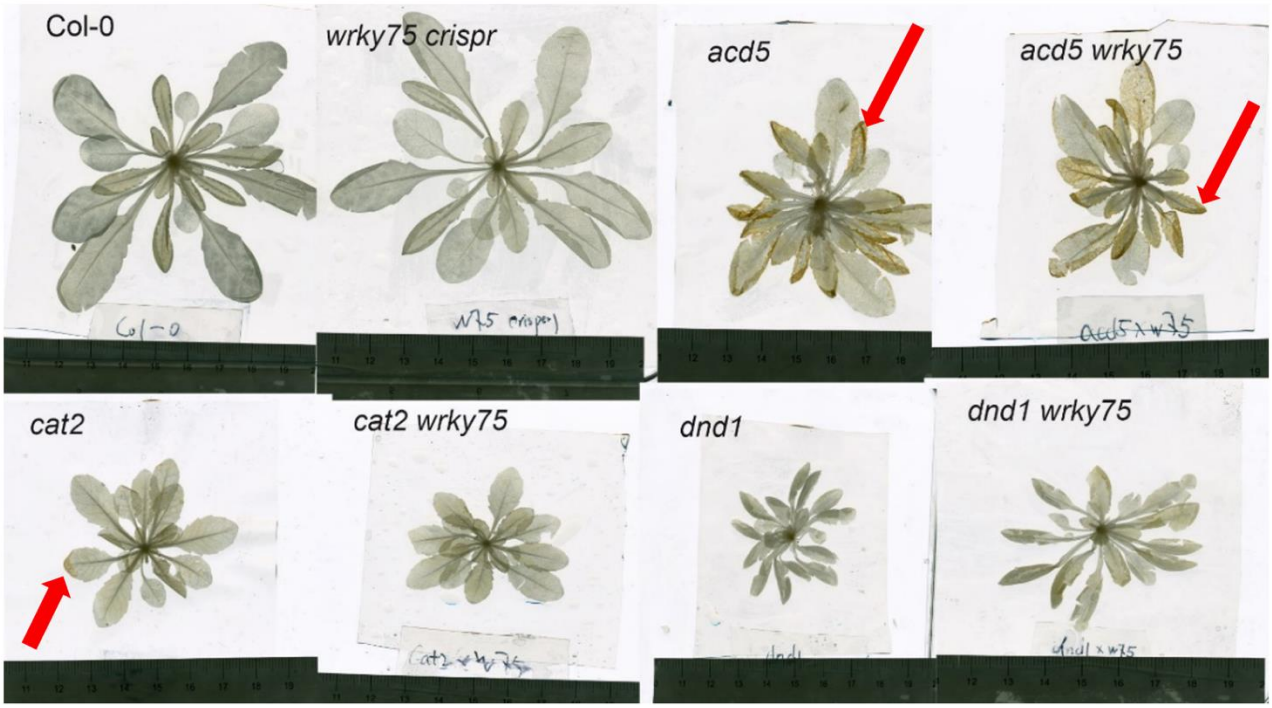

Figure. 7: DAB staining of leaves of 35-d-old Col-0, *wrky75* crispr, *acd5*, *acd5 wrky75*, *cat2*, *cat2 wrky75*, *dnd1*, *dnd1 wrky75* plants in SD condition. Brown dark areas showed H_2O_2 accumulation in plants leaves. For instance, red arrows showed the H_2O_2 accumulation in *acd5*, *acd5 wrky75* and *cat2*.

5.3 Trypan blue staining

Trypan blue staining pictures of different genotypes plants (Fig.8) showed that dark blue areas appeared in *acd5* plants and *acd5 wrky75* double mutant plants (Fig.8). The same situation happened in *dnd1* single mutant plants and *dnd1 wrky75* double mutant plants and *cat2* single mutant plants and *cat2 wrky75* double plants. There was no dark blue area in wildtype and *wrky75* single mutant.

These results indicated that cell death happened in in the lesion mimic mutants with defective cell death regulation, but not in wildtype and the *wrky75* single mutant. Additionally, the result of trypan blue staining in the *wrky75* double mutants was similar to the corresponding single mutants. For instance, there was dark blue appearance in *acd5* single mutant and *acd5 wrky75* double mutant (Fig.8).

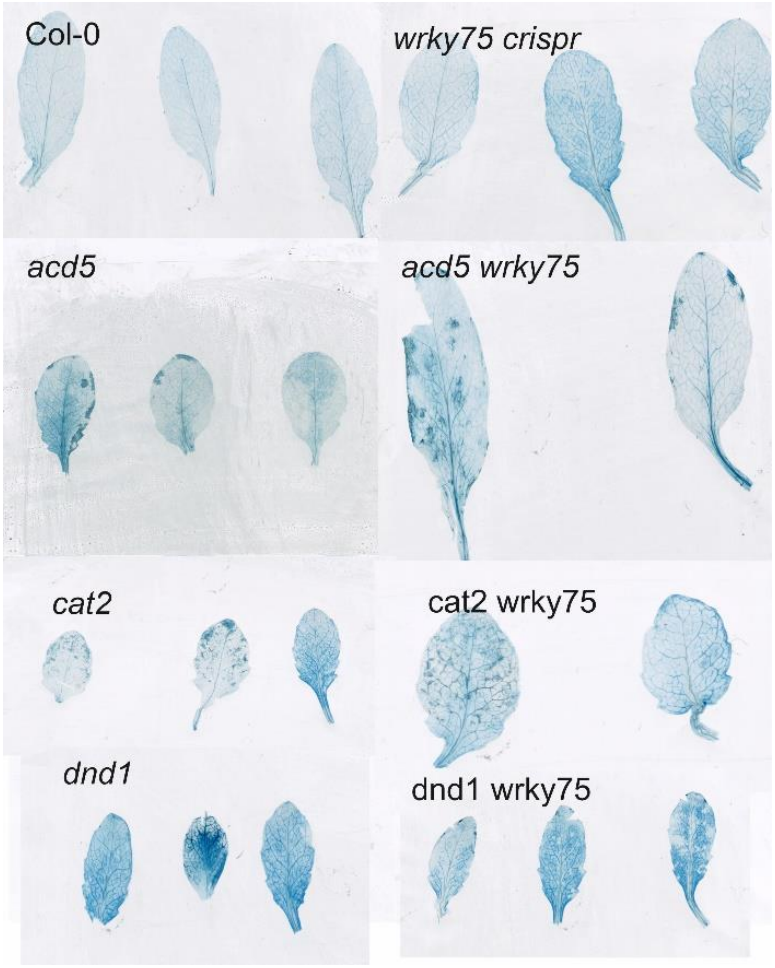

Figure. 8: Trypan blue staining of leaves of 32-d-old Col-0, *wrky75* crispr, *acd5*, *acd5 wrky75*, *cat2*, *cat2 wrky75*, *dnd1*, *dnd1 wrky75* plants grown in SD condition. Dark blue spotted in leaves and indicated dye accumulation in dead cells.

5.4 Real-time polymerase chain reaction (qPCR)

To investigate the function of *WRKY75* in regulation of gene expression, transcript levels for five marker genes was measured with qPCR both in SD and LD conditions (Fig. 9 and 10). The five marker genes were selected to be regulated during senescence and during cell death and

hormone (SA) signalling. From the SD condition results, there was no obvious difference between wildtype and *wrky75* mutant, indicating that in SD growth conditions *WRKY75* is not regulating the selected genes (Fig. 9). If *WRKY75* is acting a positive regulator of SA biosynthesis gene *ICS1* (Guo et al., 2017), it was expected to see increased *ICS1* transcript levels in *WRKY75* overexpression lines. This was not the case and instead transcript levels for *ICS1* were the same in *WRKY75* overexpression lines and in the *wrky75* crispr mutant (Fig. 9). In the three lesion mimic mutants (*acd5*, *cat2*, *dnd1*) – there was increased transcript levels for the five marker genes (although *ICS1* transcript levels were rather low). As the plants were harvested when visible cell death was seen in the lesion mimic mutants, increased transcript levels for defense-related genes was an expected result. The double mutants with *wrky75* (*acd5 wrky75*, *cat2 wrky75*, *dnd1 wrky75*), also had increased transcript levels that were similar to the corresponding single mutants (Fig. 9). Thus, the *wrky75* mutation did not influence H_2O_2 accumulation (Fig. 7), cell death (Fig. 8) or regulation of defense-related genes (Fig. 9).

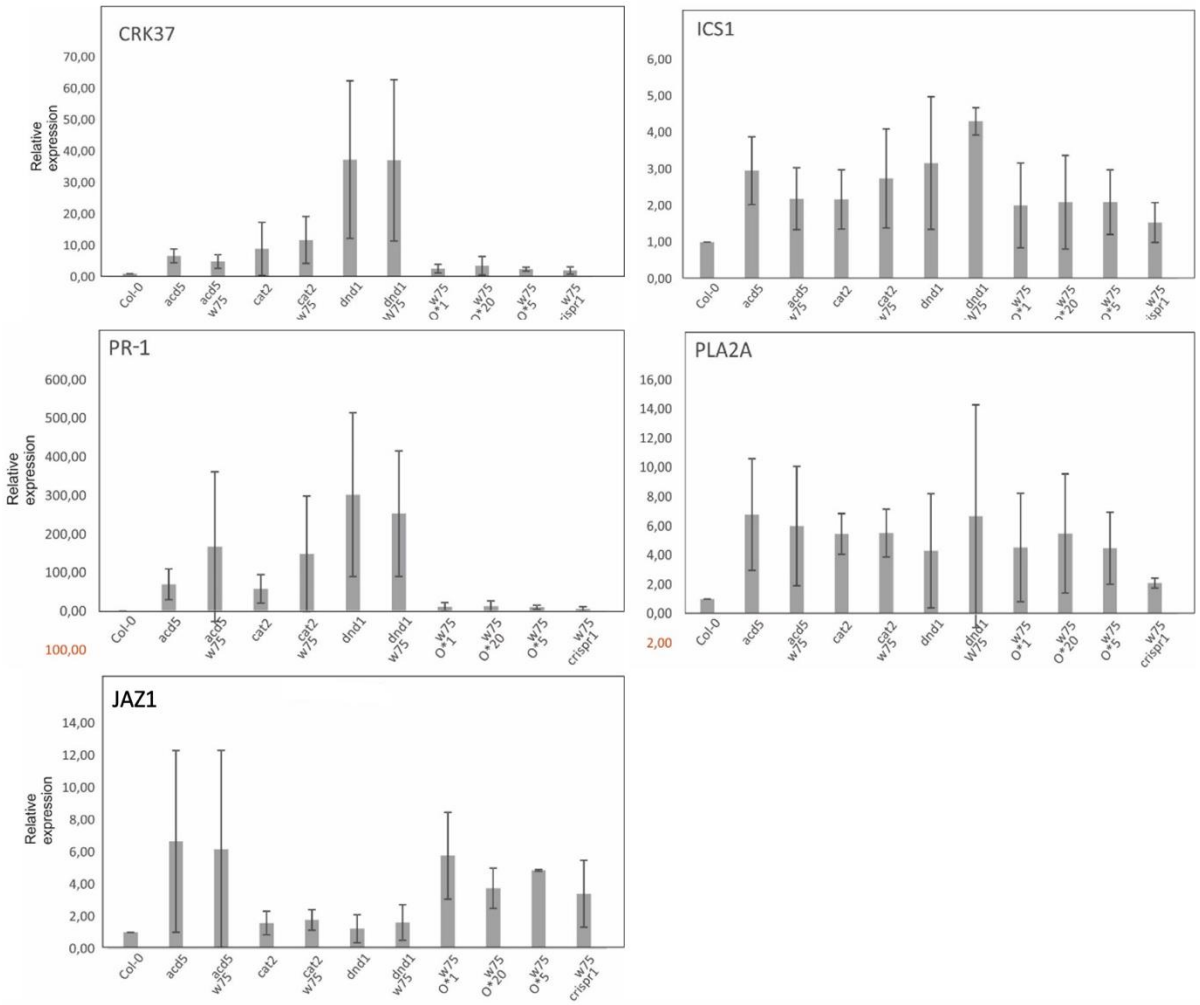

Figure.9 : qRT-PCR analysis of transcript levels of marker genes 30-d-old lesion mimic mutants and their wrky75 double mutant in short day condition by qBase+ software. Bar represents mean of three biological repeats, error bar indicates standard deviation. *YLS8*, *TIP41* and *PP2AA3* were reference genes. In addition, three different WRKY75 overexpression lines were included (O*1, O*5 and O*20).

In Guo et al. 2017, all experiments with *wrky75* were performed in LD conditions. To further characterize the single and double mutants, the growth conditions were changed from SD to LD and the qPCR experiment repeated. In LD growth condition qPCR, the *wrky75* mutant showed similar marker genes transcription levels as Col-0.

Thus, *WRKY75* also did not regulate marker genes when plants were grown in LD condition (Figure 10). For example, *ICS1* encoding one of the SA biosynthesis enzymes, had similar transcription levels in Col-0 and the *wrky75* crispr mutant. The same situation was seen for *PR-1*, a highly SA-responsive gene (Blanco, Salinas et al. 2009). Three marker genes (*CRK37, ICS1* and *PR-1*) has somewhat increased transcription levels in *WRKY75* overexpression lines, but this was inconsistent between different overexpression lines. The transcript levels for marker genes (except *PLA2A*) had increased levels in three lesion mimic mutants *acd5, dnd1* and *cat2* compared with Col-0 and *wrky75 crispr* mutant (Figure.10). However, in double mutants with *wrky75*, the transcript levels were similar as the single lesion mimic mutants. Overall, these results were the same as qPCR with SD conditions; the *wrky75* mutant did not influence regulation of five defense and senescence-regulated marker genes. Additionally, for the five marker genes, transcript levels were higher in SD condition than LD condition, especially for *PR-1* and *CRK37* (compare Figure 9 with Figure 10)**.** This indicated that SA regulation could be more important and active in SD condition than LD condition.

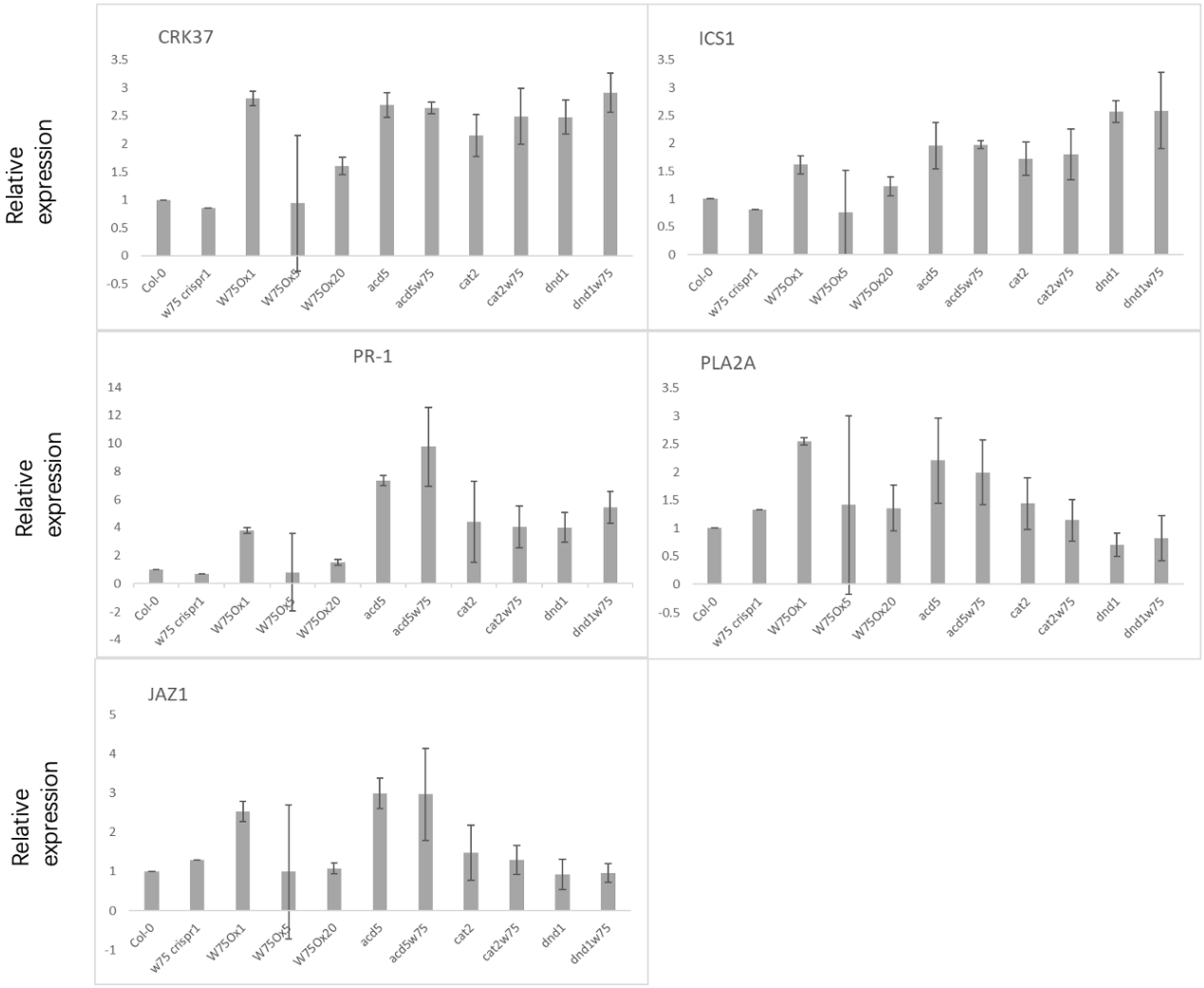

Figure.10: qRT-PCR analysis of transcript levels of marker genes in 20-d-old mimic mutant and their wrky75 double mutant in long day condition by qBase+ software. Bar represents mean of three biological repeats, error bar indicated standard deviation. *YLS8*, *TIP41* and *PP2AA3* were reference genes. In addition, three different WRKY75 overexpression lines were included (O*1, O*5 and O*20).

5.5 Botrytis infection

To evaluates the role of *WRKY75* in regulation of disease resistance, the single and double mutants were infected with Botrytis. Necrotic lesions formed on all plants. The Botrytis sensitive *coil-16* showed that the infection was successful (Figure 11).

Figure.11: Botrytis infection of JA insensitive *coi1-16*. 3 weeks and two days old plants (SD grown plants) were infected with Botrytis. Spreading lesions indicated successful infection with Botytis. Total n = 12 for *coi1-16.*

Figure.12: 3 weeks and two days old *acd5 cat2 dnd1* and their corresponding *wrky75* mutant grown in SD condition were drop-inoculated with Botrytis. Lesion diameters were quantified 48h – 72h postinfection.

The size of Botrytis lesions was analyzed. The *wrky75* single mutant had increased lesion size compared to Col-0 plants (Figure 13). $P < 0.05$ between Col-0 and *wrky75* means there is a significant difference between Col-0 and *wrky75*, indicating *WRKY75* might regulate some aspect of disease resistance. The *acd5*, *cat2*, and *dnd1* mutant single had a lower diameter of lesions than Col-0 (Figure 13). This could be a result of pre-activated defense caused by the low amount of cell death in these mutants. Interestingly there was also a significant difference between *acd5, dnd1, cat2* single mutants and their corresponding *wrky75 double* mutants (Figure 13), where lesion sizes were larger in the double mutants. Again, this indicated that WRKY75

might be a positive regulator of botrytis resistance.

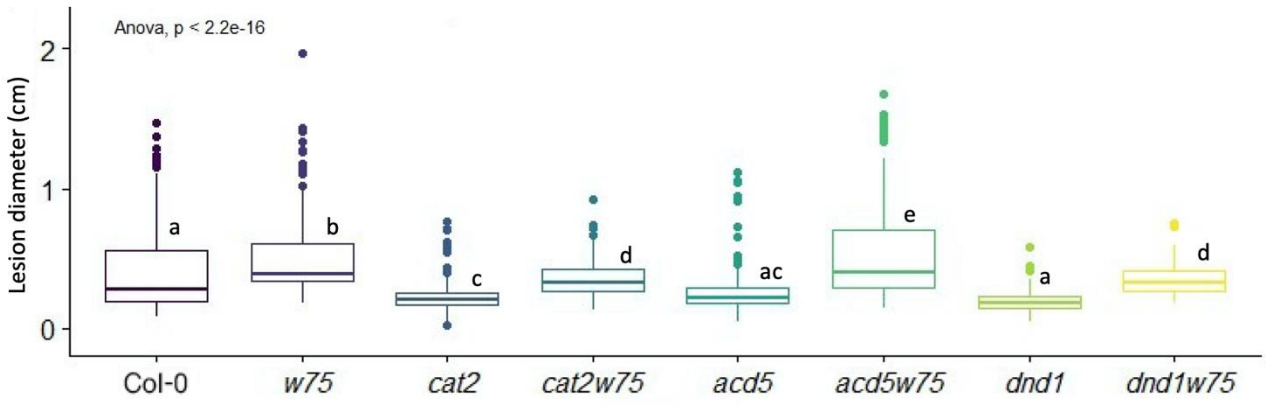

Figure 13: The size of Botrytis lesions from drop-infected leaves. Box plot was used to display the data from three biological repeats. Total n= 12 per genotype. Letters above box plots indicate significance groups among genotypes (Mixed linear model, Anova test, P< 0.05). Botrytis lesion diameters were quantified 48 h-72 h after infection. See **Appendix 2** for the whole statistical analysis of botrytis infection.

5.6 Kanamycin and hygromycin selection of WRKY75-SRDX lines.

To generate homozygote lines which contian *SRDX- WRKY75* chimeric repressor, the floral dip method was used to transfer the transgenes to Arabidopsis (Figure.14)

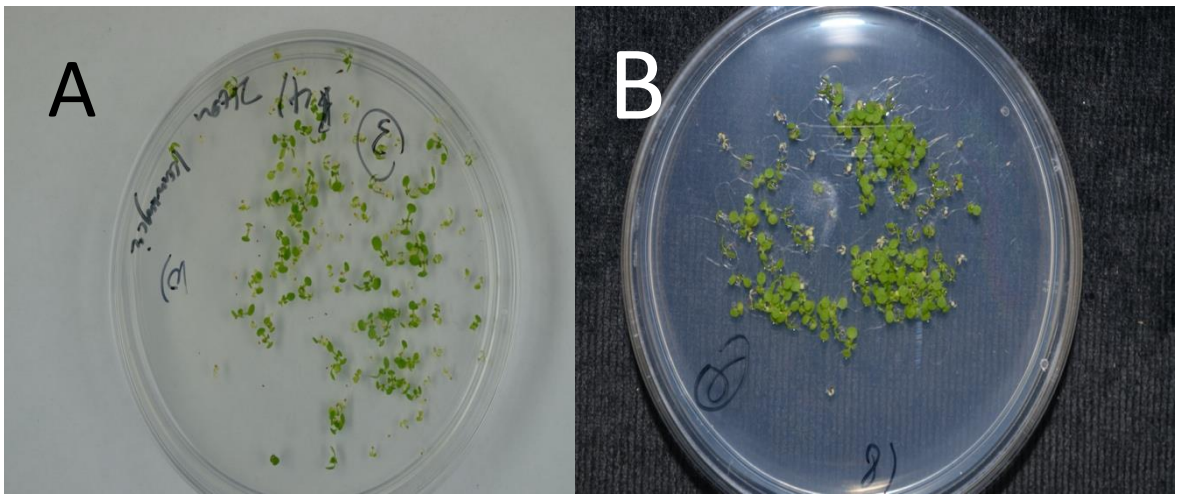

Figure.14: An example of T2 selection seeds. A, seeds were plated on 1% agar comprising MS medium and kanamycin (50 mg/ ml) . B, seeds were plated on 1% agar comprising MS medium and hygromycin (20 mg/ ml). Long green hypocotyls indicate hygromycin resistant plant.

Supplementary data 4 and 5 have T2 generation kanamycin and hygromycin selection data (Figure 14.). Lines were chosen where the number of resistant seedlings: sensitive seedlings was about 3:1. Subsequently, qPCR was performed in T2 generation lines to analyze *SRDX-WRKY75* chimeric repressor expression level in ozone and control conditions (Figure 15). *PP2AA3* and *TIP41* were used as reference gene and Col-0 and *wrky75* were controls. For future work, lines in which ozone treatment *WRKY75-SRDX* transcript levels were highest and had a large difference with control treatment were chosen and different markers genes related to *WRKY75* such as *ICS1* were chosen to analyze the marker genes transcription level in chosen lines.

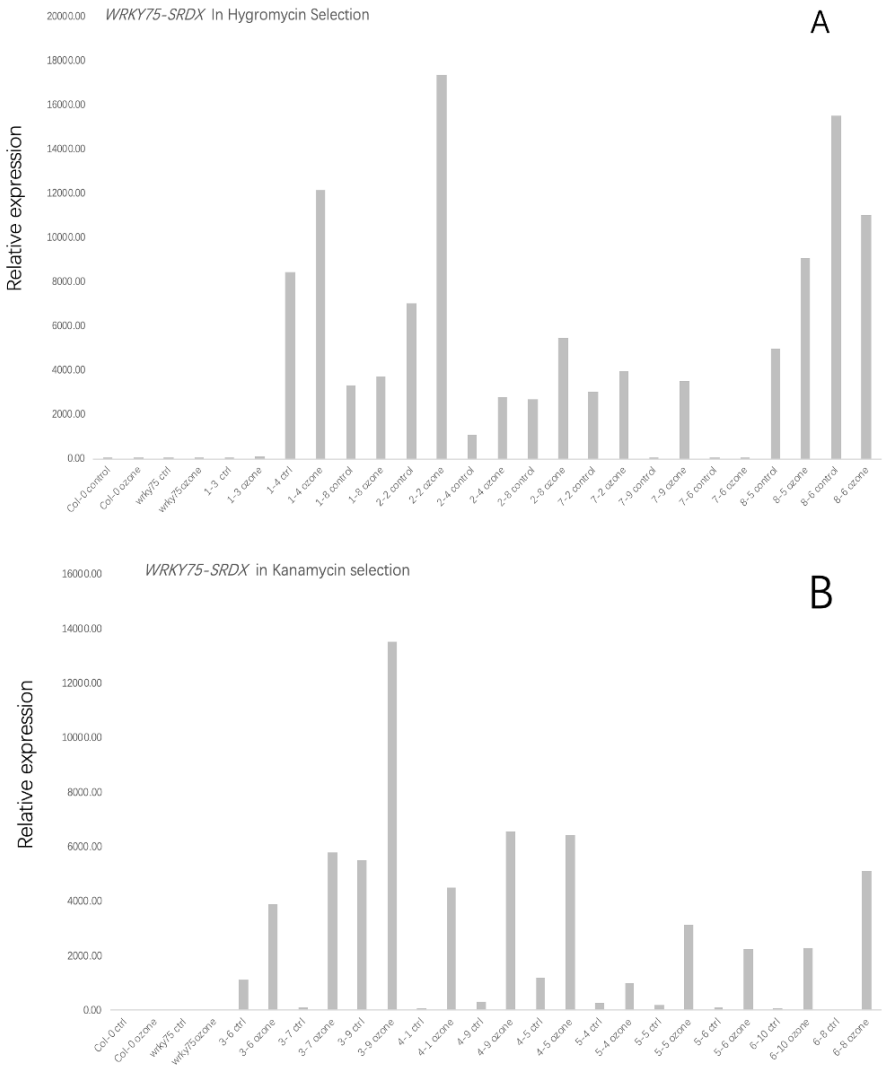

Figure. 15: qRT-PCR analysis of transcript levels of *SRDX-WRKY75* chimeric repressor in 20-d-old in SD condition by qBase+ software. A, *SRDX-WRKY75* transcription level in Hygromycin selection lines. B, *SRDX-WRKY75* transcription level in Kanamycin selection lines. *PP2AA3* and *TIP41* were used as reference gene and Col-0 and *wrky75* were controls

6. Discussion

Leaf senescence is a highly coordinated particular genetically regulated process existing in activation of precisely controlled signalling pathways which result in cell suicide (Bruggeman, Raynaud et al. 2015). This process requires the integration of a wide variety of internal and environmental signals (Woo, Kim et al. 2013). Additionally, SA and ROS are inducers of leaf senescence and also cell death (Lim, Kim et al. 2007). According to previous viewpoints, WRKY75 transcription factor can regulate leaf senescence in Arabidopsis positively. Knockout or knockdown this gene can reduce the process of leaf senescence. Meanwhile, overexpression of this gene can promote leaf senescence. Additionally, a tripartite amplification loop with the transcription factor WRKY75, SA and ROS can accelerate leaf senescence, and they can affect each other (Guo, Pengru, et al. 2017).

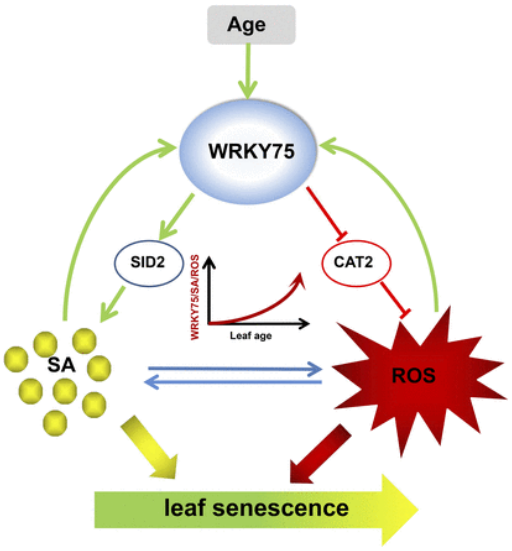

Figure.16: The tripartite amplification loop from Guo et al., 2017, which suggest an amplification loop between WRKY75, salicylic acid and ROS in regulation of senescence.

This tripartite amplification loop indicates that WRKY75 can promote the production of SA via increased *ICS1/SID2* gene transcription. At the same time, WRKY75 can repress *CAT2* gene transcription to active H_2O_2 production which can accelerate senescence and cell death (Fig.16). In principle, if we knockout *WRKY75* gene or repress the *WRKY75* transcription, the SA and H_2O_2 production should be reduced which can lead to delay of leaf senescence (cell death).

To ensure correct results in this thesis work, the lesion mimic mutants *acd5, dnd1* and their corresponding *wrky75* double mutant were first genotyped with dCAPS markers to confirm that correct mutations were present. Cleaved amplified polymorphic sequence (CAPS) and dCAPS markers are used for detection of Single Nucleotide Polymorphism (SNP) (Konieczny and Ausubel 1993, Neff, Neff et al. 1998). CAPS relies on the use of restriction enzymes that cut the sequence where the SNP is present. However, when the mutation (SNP) does not locate at a restriction enzyme site, a CAPS marker cannot be used. The advantage of dCAPS is that it can include a mutation site in the dCAPS primer, which can create a new restriction enzyme site even though no restriction enzyme site was present before. For *cat2* (which is a T-DNA mutant), LP primer, Lba primer and RP primer were used for PCR.

The goal of this work was to further investigate the function of transcription factor WRKY75 in regulation of cell death and defense gene expression. However, there was similar H_2O_2 accumulation and cell death in both *acd5, dnd1, cat2* single mutants and their corresponding *wrky75* double mutants *acd5 wrky75*, *dnd1 wrky75* and *cat2 wrky75* by DAB staining experiment and trypan blue staining (Figs. 7 and 8). The double mutants a*cd5 wrky75, dnd1 wrky75, cat2 wrky75* phenotypes were not different from the single mimic mutants. Additionally, the phenotype of *wrky75* crispr mutant was the same as wildtype in experiment DAB staining. This indicated that WRKY75 transcription factor might not regulate H_2O_2 accumulation or cell death in these mutant plants. There was some cell death in *cat2* mutant and *cat2 wrky75* mutant. The function of CATALASE2 is to scavenge H_2O_2 accumulation. Consequently, in the knockout *cat2*, the H_2O_2 should accumulate which was correspondence to the results obtained here.

To complement results from DAB staining and Trypan blue staining experiment, qPCR was used to measure transcription levels in SD and LD conditions. SD growth condition was used first, as these are the standard growth conditions in the Helsinki Arabidopsis growth rooms. Subsequently, to match the growth conditions in Guo et al., 2017, also LD growth conditions were used. For qPCR marker genes related to hormone signalling, cell death and senescence

were used. Similarly, to DAB and trypan blue staining, no obvious marker gene transcript level differences were observed between *acd5, cat2, dnd1* and their corresponding double mutant mutants *acd5 wrky75, dnd1 wrky75* and *cat2 wrky75*. Additionally, marker gene transcript levels in wildtype were similar as in *wrky75* crispr mutant. This indicated that WRKY75 transcription factor might not play a function in cell death or SA synthesis regulation in these mutants in SD conditions.

Looking closer at specific marker genes, high transcript levels was seen for *CRK37* in *dnd1* and *dnd1 wrky75* lines (Figure .9). As no difference between the single and double mutants was observed, this indicated that the expression of *CRK37* is not regulated by WRKY75. Cell death in the *dnd1* mutant can induce the accumulation of SA defense related genes expression (Xu and Brosché 2014). This could explain the high level of CRK37 in *dnd1* and *dnd1 wrky75*.

Another marker gene used was *PR-1*, a defense-related gene in Arabidopsis. SA strongly increase *PR-1* transcript levels, and *PR-1* is one of the most usually marker genes can be used to show activation of SA signalling (Glazebrook 2001)**.** Cell death happened in all lesion mimic mutants. This could be the reason *PR-1* had a higher expression in lesion mimic lines. The results clearly showed *PR-1* transcript levels increased more in *acd5 wrky75* mutant lines than *acd5* lines (Fig.9). Increased *PR-1* transcript levels in *acd5 wrky75* might mean increased SA accumulation in *acd5 wrky75* plants than *acd5*. This result was opposite to our excepted result (that is, if WRKY75 is a positive regulator lower transcript levels or SA accumulation should be seen in the double mutant).

As the suggested role for WRKY75 as a positive regulator (Guo et al., 2017), could not be seen in the experiments performed with DAB, trypan blue or qPCR in SD growth conditions, this needed an explanation. It could be a result of genetic redundancy or different growth conditions. Guo et al, used a *WRKY75* RNAi line that might have resulted in impaired function in several WRKY transcription factors (assuming that they share some sequence similarity). The target gene identification is always interfered by a redundancy of genes for plant transcription factors (Hiratsu, Matsui et al. 2003). There might be other *WRKY75* homolog genes playing a role of *ICS1* regulation and SA synthesis. Also, Arabidopsis was grown in SD conditions (12h-light /12h-dark cycle, 22 ℃/19 ℃, and relative humidity of 50%/60%) which was different with the growth condition in in Guo et al. 2017, which used a 16-h-light/8-h-dark cycle, 22 ℃. Hence, the qPCR experiment was repeated in LD growth condition plants with the same marker genes.

Similarly, to SD qPCR, no obvious marker gene transcript level differences were observed between *acd5, cat2, dnd1* and their corresponding *wrky75* double mutant mutants also between Col-0 and *wrky75* crispr mutant in LD growth condition. This indicated that WRKY75 also did not regulate defense gene expression in LD growth conditions. Consistent between SD and LD growth conditions, was also elevated expression of the marker genes in lesion mimic mutants. This could be a consequence of these plants undergoing cell death leading to activation of defense gene expression.

According to published data, WRKY75 is a positive regulator of disease resistance in Arabidopsis through the promotion of SA accumulation, which is required for many diseases resistance (Dong 1998) (Guo, Li et al. 2017) (Greenberg, Silverman et al. 2000). To investigate if WRKY75 regulate disease resistance, Botrytis infection was performed in the lesion mimic mutants and their corresponding *wrky75* double mutant also *wrky75* crispr single mutant. Significant differences were observed between *acd5, cat2, dnd1* and corresponding *wrky75* double mutants. Also, a significant difference was shown between Col-0 and *wrky75* crispr single mutant (Figure.13). In all cases, genotypes with *wrky75* had larger lesions. This indicated that WRKY75 positively regulated disease resistance in Arabidopsis. More SA accumulation may be present in *acd5, cat2, and dnd1* compared with corresponding *wrky75* double mutants, but this requires further experiments for verification. Smaller lesion sizes in the lesion mimic single mutants could be explained by pre-activated defense programs caused by cell death.

Another reason for the lack of phenotype in *wrky75* single and double mutants could be genetic redundancy. WRKY transcription factors belong to a large gene family and possibly some other WRKY transcription can act redundantly with WRKY75. In this context, it is important to notice that Guo et al., 2017, used an WRKY75 RNAi line in many experiments, this line might accidentally have more than WRKY75 silenced (that is, one or more WRKY transcription factors with sequence similarity to the RNAi construct). To overcome genetic redundancy, new transgenic lines with WRKY75 fused to a strong transcriptional repressor SRDX was constructed in this thesis (Figures 5 and 15). These can be used in future work to further define the role of WRKY75 in regulation of SA biosynthesis, cell death and other defense responses.

7. Conclusion and future work:

In summary, the expected results as indicated by Guo et al., 2017 was not obtained. WRKY75 was suggested to be a positive regulator of SA signalling and cell death. Results in this thesis indicated that there were no different phenotypes (H_2O_2 accumulation and cell death) between Col-0 and *wrky75* crispr mutant as well as *acd5, cat2, dnd1* and their corresponding *wrky75* double mutants. The marker genes transcript levels gave similar results. i.e. no clear differences between single and double mutants. Thus, no evidence was obtained that could prove the function of WRKY75 transcription factor as a regulator of synthesis of SA and cell death. Additionally, marker genes transcription level in SD qPCR were much higher compared with LD qPCR which indicated that SA synthesis and cell death were more positive regulated in SD growth condition. The Botrytis infection result indicated WRKY75 could be a positive regulator of plant disease resistance.

There might be gene redundancy for SA synthesis and *ISC1* regulation. There might be some other *WRKY75* homologues genes regulate this complicated process together with *WRKY75*. To eliminate gene redundancy in *WRKY75*, *SRDX-WRKY75* lines were generated. In these lines WRKY75 acts as a dominant repressor to suppress all target genes of WRKY75 even in the presence of other *WRKY75* homologues genes (Hiratsu, Matsui et al. 2003). With these new lines, the transcription level of marker genes related to hormone signalling, cell death, senescence and especially the SA biosynthesis *ISC1* would be analyzed to evaluate the function of *WRKY75*.

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9. Supplementary data

Supplementary data 1	
10xbuffer	2
DNAseI	1
RNAse inhibiotor	0,1
H_2O	1,8
totally	3,25

Supplementary data 2	
oligo-dT	1
5xbuffer	6,2
dNTP	1,5
Maxima reverse transcriptase	0,5
RNAse inhibitor	0,1
H_2O	0,15
totally	9,45

Supplementary data 3			
Gene AgI code	Gene annotation	Amplification efficience	Primer sequence
At4g04500	CRK37	1.631	CCAGAATACGCGACATACGGACAAT, CAATCCACCTCTTCCACACAAAAGC
At2g14610	PR-1	1.658	TGATCATGCATACACACGTACA, CATCCTGCATATGATGCTCCT
At1g74710	ISC1/SID2	1.928	GCTTGGCTAGCACAGTTACAGC, CACTGCAGACACCTAATTGAGTCC
At1g19180	JAZ1	1.801	TGCGATCCAGCCAAAGCGTC, TTGGTACGGCTTGAGGGTGGT
At2g26560	PHOSPHPLIPASE A 2A	1.778	CGGGTTGTAACGAAAATGCT, TGCATGAGGTGAACGAATGT
At5g08290	YLS8	1.822	CACTGAATCATGTTCGAAGCAAGT, GCGGTTGTGGAGAACATGATACG
At1g13320	PP2AA3	1.854	GCGGTTGTGGAGAACATGATACG GAACCAAACACAATTCGTTGCTG
At4g34270	TIP41	1.867	GTGAAAACTGTTGGAGAGAAGCAA, TCAACTGGATACCCTTTCGCA

Supplemantary data 4					
hygromycin selection lines	number	resistance	sensitive	proportion	line description
1	1	131	53	0.404580153	(wrky75)WRKY75 cDNA +WT SRDX
1	2	115	52	0.452173913	(wrky75)WRKY76 cDNA +WT SRDX
1	3	91	28	0.307692308	(wrky75)WRKY77 cDNA +WT SRDX
1	4	78	22	0.282051282	(wrky75)WRKY78 cDNA +WT SRDX
1	5	211	44	0.208530806	(wrky75)WRKY79 cDNA +WT SRDX
1	6	102	27	0.264705882	(wrky75)WRKY80 cDNA +WT SRDX
1	7				(wrky75)WRKY81 cDNA +WT SRDX
1	8	75	25	0.333333333	(wrky75)WRKY82 cDNA +WT SRDX
1	9	136	45	0.330882353	(wrky75)WRKY83 cDNA +WT SRDX
1	10	38	more than 100		(wrky75)WRKY84 cDNA +WT SRDX
hygromycin selection	number	resistance	sensitive	proportion	
2	1	97	42	0.432989691	(Col-0)WRKY75 cDNA +WT SRDX
2	2	122	38	0.31147541	(Col-0)WRKY76 cDNA +WT SRDX
2	3	0			(Col-0)WRKY77 cDNA +WT SRDX
2	4	73	28	0.383561644	(Col-0)WRKY78 cDNA +WT SRDX
2	5	80	15	0.1875	(Col-0)WRKY79 cDNA +WT SRDX
2	6	75	31	0.413333333	(Col-0)WRKY80 cDNA +WT SRDX
2	7	0			(Col-0)WRKY81 cDNA +WT SRDX
2	8	188	50	0.265957447	(Col-0)WRKY82 cDNA +WT SRDX
2	9	105	22	0.20952381	(Col-0)WRKY83 cDNA +WT SRDX
2	10	187	16	0.085561497	(Col-0)WRKY84 cDNA +WT SRDX
hygromycin selection	number	resistance	sensitive	proportion	
7	1	75	22	0.293333333	(wrky75) WRKY75 cDNA +mutant SRDX
7	2	105	29	0.276190476	(wrky75) WRKY75 cDNA +mutant SRDX
7	3	101	17	0.168316832	(wrky75) WRKY75 cDNA +mutant SRDX
7	4	0			(wrky75) WRKY75 cDNA +mutant SRDX
7	5	76	19	0.25	(wrky75) WRKY75 cDNA +mutant SRDX
7	6	105	29	0.276190476	(wrky75) WRKY75 cDNA +mutant SRDX
7	7	2	almost 190		(wrky75) WRKY75 cDNA +mutant SRDX
7	8	141	20	0.141843972	(wrky75) WRKY75 cDNA +mutant SRDX
7	9	125	40	0.32	(wrky75) WRKY75 cDNA +mutant SRDX
7	10	18	98	5.444444444	(wrky75) WRKY75 cDNA +mutant SRDX
hygromycin selection	number	resistance	sensitive	proportion	
8	1	67	19	0.28358209	(Col-0)WRKY75 cDNA +mutant SRDX
8	2	1		0	(Col-0)WRKY75 cDNA +mutant SRDX
8	3	0			(Col-0)WRKY75 cDNA +mutant SRDX
8	4	115	30	0.260869565	(Col-0)WRKY75 cDNA +mutant SRDX
8	5	73	28	0.383561644	(Col-0)WRKY75 cDNA +mutant SRDX
8	6	133	38	0.285714286	(Col-0)WRKY75 cDNA +mutant SRDX
8	7	15	116	7.733333333	(Col-0)WRKY75 cDNA +mutant SRDX
8	8	75	18	0.24	(Col-0)WRKY75 cDNA +mutant SRDX
8	9	148	17	0.114864865	(Col-0)WRKY75 cDNA +mutant SRDX

Supplementary data 5					
Kanamycin selection	number	resistance	sensitive	proportion	lines deacription
3	1	147	39	3.76923	(wrky75)WRKY75 genomic +WT SRDX
3	2	25	22	1.13636	(wrky75)WRKY75 genomic +WT SRDX
3	3	62	54	1.14815	(wrky75)WRKY75 genomic +WT SRDX
3	4	84	32	2.62500	(wrky75)WRKY75 genomic +WT SRDX
3	5	69	19	3.63158	(wrky75)WRKY75 genomic +WT SRDX
3	6	115	39	2.94872	(wrky75)WRKY75 genomic +WT SRDX
3	7	114	37	3.08108	(wrky75)WRKY75 genomic +WT SRDX
3	8	168	38	4.42105	(wrky75)WRKY75 genomic +WT SRDX
3	9	100	36	2.77778	(wrky75)WRKY75 genomic +WT SRDX
3	10	81	41	1.97561	(wrky75)WRKY75 genomic +WT SRDX
Kanamycin selection	number	resistance	sensitive	proportion	
4	1	85	30	2.83333	(Col-0)WRKY75 genomic +WT SRDX
4	2	78	72	1.08333	(Col-0)WRKY75 genomic +WT SRDX
4	3	99	43	2.30233	(Col-0)WRKY75 genomic +WT SRDX
4	4			#DIV/0!	(Col-0)WRKY75 genomic +WT SRDX
4	5	100	31	3.22581	(Col-0)WRKY75 genomic +WT SRDX
4	6	83	21	3.95238	(Col-0)WRKY75 genomic +WT SRDX
4	7		6	0.00000	(Col-0)WRKY75 genomic +WT SRDX
4	8		3	0.00000	(Col-0)WRKY75 genomic +WT SRDX
4	9	92	26	3.53846	(Col-0)WRKY75 genomic +WT SRDX
4	10		5	0.00000	(Col-0)WRKY75 genomic +WT SRDX
Kanamycin selection	number	resistance	sensitive	proportion	
5	1	174	69	2.52174	(wrky75) WRKY75 genomic +mutant SRDX
5	2	128	25	5.12000	(wrky75) WRKY75 genomic +mutant SRDX
5	3	123	43	2.86047	(wrky75) WRKY75 genomic +mutant SRDX
5	4	85	27	3.14815	(wrky75) WRKY75 genomic +mutant SRDX
5	5	110	37	2.97297	(wrky75) WRKY75 genomic +mutant SRDX
5	6	98	32	3.06250	(wrky75) WRKY75 genomic +mutant SRDX
5	7	86	36	2.38889	(wrky75) WRKY75 genomic +mutant SRDX
5	8			#DIV/0!	(wrky75) WRKY75 genomic +mutant SRDX
5	9	78	12	6.50000	(wrky75) WRKY75 genomic +mutant SRDX
5	10	80	23	3.47826	(wrky75) WRKY75 genomic +mutant SRDX
Kanamycin selection	number	resistance	sensitive	proportion	
6	1	96	30	3.20000	(Col-0)WRKY75 genomic +mutant SRDX
6	2	139	51	2.72549	(Col-0)WRKY75 genomic +mutant SRDX
6	3	85	34	2.50000	(Col-0)WRKY75 genomic +mutant SRDX
6	4	114	31	3.67742	(Col-0)WRKY75 genomic +mutant SRDX
6	5	94	21	4.47619	(Col-0)WRKY75 genomic +mutant SRDX
6	6	92	28	3.28571	(Col-0)WRKY75 genomic +mutant SRDX
6	7	76	20	3.80000	(Col-0)WRKY75 genomic +mutant SRDX
6	8	84	28	3.00000	(Col-0)WRKY75 genomic +mutant SRDX
6	9	110	37	2.97297	(Col-0)WRKY75 genomic +mutant SRDX
6	10	100	36	2.77778	(Col-0)WRKY75 genomic +mutant SRDX

Appendix 1
The layout of the 384 wells plate for qPCR

Primer	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
CRK37																								
ICS1																								
PLA2A																								
PR-1	Col-0			*wrky75*			*acd5*			*acd5 wrky75*			*dnd1*			*dnd1 wrky75*			*cat2*			*cat2 wrky75*		
JAZ1																								
PP2AA3																								
TIP41																								
YLS8																								
CRK37																								
ICS1																								
PLA2A																								
PR-1	*WRKY75* overexpressionx1			*WRKY75* overexpression x5			*WRKY75* overexpression x20																	
JAZ1																								
PP2AA3																								
TIP41																								
YLS8																								

Appendix 2

Statistical analysis of botrytis infection